INTERACTION BETWEEN PSORALEN AND TYROSINASE IN PRESENCE AND ABSENCE OF U V LIGHT

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CERTIFICATE

I certify that the work presented in the following pages has been carried out by Mr. Sohel Talib and that it is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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DEDICATION

Dedicated to my parents.

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ABSTRACT

The process of melanin formation in mammalian skin is known to be catalysed by tyrosinase. In vitiligo, psoralen administration followed by exposure to radiant energy, enhances the formation of pigment. However, the mode of action of psoralen in UV induced skin pigmentation remains to be clearly understood. In view of the above fact, the effect of UV irradiation on tyrosinase in presence and absence of psoralen was studied. A systematic study on the conformation and biological activity of tyrosinase in presence of psoralen was also investigated. A detailed study of different inhibitors on tyrosinase has been carried out to correlate their destruction by UV light and consequent release of tyrosinase activity.

Decrease in fluorescence intensity of tyrosinase in presence of psoralen was taken as a measure of interaction. The association constant was determined to be $8.4 \times 10^6\ M^{-1}$; the value of free energy required to stabilise the psoralen-tyrosinase complex was computed to be -9 Kcal/mole, which convinsingly indicated that the complex between psoralen and tyrosinase is quite stable. Since psoralen is hydrophobic in nature, decrease in enzyme fluorescence in presence of psoralen suggests the possible involvement of aromatic amino acid residues in its interaction with tyrosinase. The molar-ratio of psoralen-tyrosinase system was found to be pH dependent; maximum (77) being at pH 6.5,

suggests the possible involvement of histidine residues in stabilization of psoralen-enzyme complex.

The dual activity of tyrosinase as a function of psoralen concentration was measured and a significant increase in both the activities was observed. It may be suggested that psoralen acts as a stimulator of cresolase and catecholase activities of tyrosinase. The salt concentration from 0.01 to 0.1 M does not influence the fluorescence of psoralen-enzyme complex. The involvement of hydrophobic and short range electrostatic interaction is suggested.

Effect of a potent denaturant, urea on the conformation and biological activity of tyrosinase was studied and at 8 M urea concentration complete loss in biological activity was noticed. In presence of psoralen the effect of the denaturant on biological activity as well as on the structure of enzyme molecule was considerably minimised indicating a protective role of psoralen.

Since UV irradiation in presence of psoralen potentiates the tyrosinase activity, the effect of different doses of longwave UV light on psoralen, tyrosinase and psoralen-tyrosinase complex was studied. The relative fluorescence of tyrosinase was increased with increase in irradiation dose which may be attributed to the unfolding of the molecule. Tyrosinase, irradiated in presence of psoralen does not show any increase in fluorescence. It is inferred from this observation that psoralen is not acting as a photosensitiser in the present system. As a result of irradiation, psoralen shows a significant shift in its characteristic maxima and development of a new well defined peak, the fluorescence intensity of which increased with increasing irradiation dose.

The effect of UV light on the biological activity of tyrosinase in presence of psoralen was also investigated. Irradiation in presence of psoralen lowers the activity at a comparatively faster rate and it follows first order kinetics. The relatively higher rate of inactivation of enzyme in presence of psoralen might be due to increase in binding of psoralen with enzyme molecule. This is supported by the molar ratio of psoralen-tyrosinase system which was increased from 77 (in absence of UV light) to 92. Increase in binding of psoralen alongwith UV light most probably induced some change in the local conformation very near to active site, which resulted in the decreased enzyme activity.

Determination of Km values for tyrosine and dopa in presence and absence of UV light favours two independent active sites in the enzyme molecule. The radiation induced decrease in Km implies that the irradiated enzyme binds the substrate more strongly than does the non-irradiated tyrosinase. Similar observations have been reported recently with irradiated lactic dehydrogenase.

A decrease in the extent of inhibition by thiol compounds on irradiated tyrosinase was observed. This might be due to certain modification in the conformation of enzyme molecule as a result of irradiation, however, the extent of inhibition after irradiation in presence of psoralen was not affected to an appreciable extent. The Ki value for thiouracil and glutathione was found to be increased after irradiation of enzyme, however, the Ki for thiouracil in presence of psoralen followed by UV irradiation was not significantly influenced. The results favour the postulate that psoralen protects the enzyme molecule from the damaging effects of radiation in such a way that the

association constant for enzyme and inhibitor is not affected.

Inspite of the speculation that UV irradiation photooxidises the sulfhydryl compounds (tyrosinase inhibitors) and releases the tyrosinase activity leading to increased pigmentation, the results of present study strongly showed that the photooxidised inhibitors were equally potent to inhibit the enzyme activity. A marked change observed in their UV absorption spectra on exposure to UV light, suggested some modification in the configuration of these inhibitors (thiouracil and diethyldithiocarbamate). The extent of modification was pronounced on irradiation in presence of psoralen.

The results of polyacrylamide gel electrophoresis of native and irradiated tyrosinase in presence and absence of psoralen, indicated an increase in net negative charge on the protein molecule. Further conformational change in enzyme molecule on irradiation in presence and absence of psoralen was studied by tryptic digestion. The tryptic digestion of tyrosinase was increased on UV exposure, however, irradiation in presence of psoralen minimised the tryptic digestion. This further strengthen the protective effect of psoralen on tyrosinase from UV irradiation.

Immunological studies showed that the irradiation in presence and absence of psoralen induced a conformational change in the tyrosinase thereby a complete loss in the immunological activity was observed. However, the biological activity was retained to 30 per cent under similar conditions indicated that the antigenic determinants and active site are situated apart on the protein molecule.

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I. INTRODUCTION

The colour of the skin has given rise to many unfortunate trends in politics and society. The most prevalent disease associated with skin colour is vitiligo. One of the recent studies showed that the incidence of vitiligo is maximum in India (79).

Vitiligo refers to idiopathic depigmentation of human subjects which begins after birth and is usually progressive (26,57,63,68,69, 114). Very little is known about the pathogenesis of the disease. According to Lerner (69), the disorder is associated with the activity of the peripheral nerve endings in the skin. Biochemical studies (69) on vitiliginous subjects showed no abnormality in urinary noradrenaline secretion, 17-ketosteroid and M S H excretion, gastric analysis before and after histamine administration (118). The serum protein bound iodine, I^{131}, uptake by thyroid gland, basal metabolic rate, serum copper, thymol turbidity, cephalin cholesterol flocculation and skin temperature were reported to be almost normal in vitiliginous subjects (20).

The term melanin is used to denote various shades of brown and black pigments occurring naturally in mammals. Melanin pigment results

from the plymerization of oxidation products of orthodihydroxyphenyl compounds to insoluble substances of high molecular weight (74). Melanin derived from mammalian tissue is always bound to a protein and present as a particulate. According to the electron microscopic and biochemical studies of Seiji et al. (135), it is now known to occur in two forms: as melanosomes - enzymically active particles which are the site of melanin formation and are located within the cytoplasm of the melanocyte, and as melanin granules - heavily pigmented particles without tyrosinase activity and is no longer necessarily confined to the cytoplasm of the melanocyte. In other words melanogenesis is an irreversible process in which an enzymically active particle (the melanosome) is formed and gradually transformed into a mass of inert melanoprotein (the melanin granule) which is incapable of further melanin formation.

Melanin formation involves the conversion of the colourless, naturally occurring amino acid, tyrosine, to a insoluble brown polymer. This process can be carried out in the mammalian tissue only by the catalytic action of the enzyme tyrosinase. Enzymatic steps involved in melanin formation have been most successfully investigated in plants and fungi. Inspite of the fact that tyrosinase was found to be widely distributed in nature, (its existance was reported in numerous plants, bacteria, invertebrates and vertebrates) contradictory reports appeared for a long time about the presence of tyrosinase in melanin forming mammalian tissues (98,115,127). The confusion was once for all cleared by the studies of Hogeboom and Adams (50) who found transplantable mouse melanoma to contain tyrosinase. Later the presence of tyrosinase activity was demonstrated in normal human skin that had been irradiated

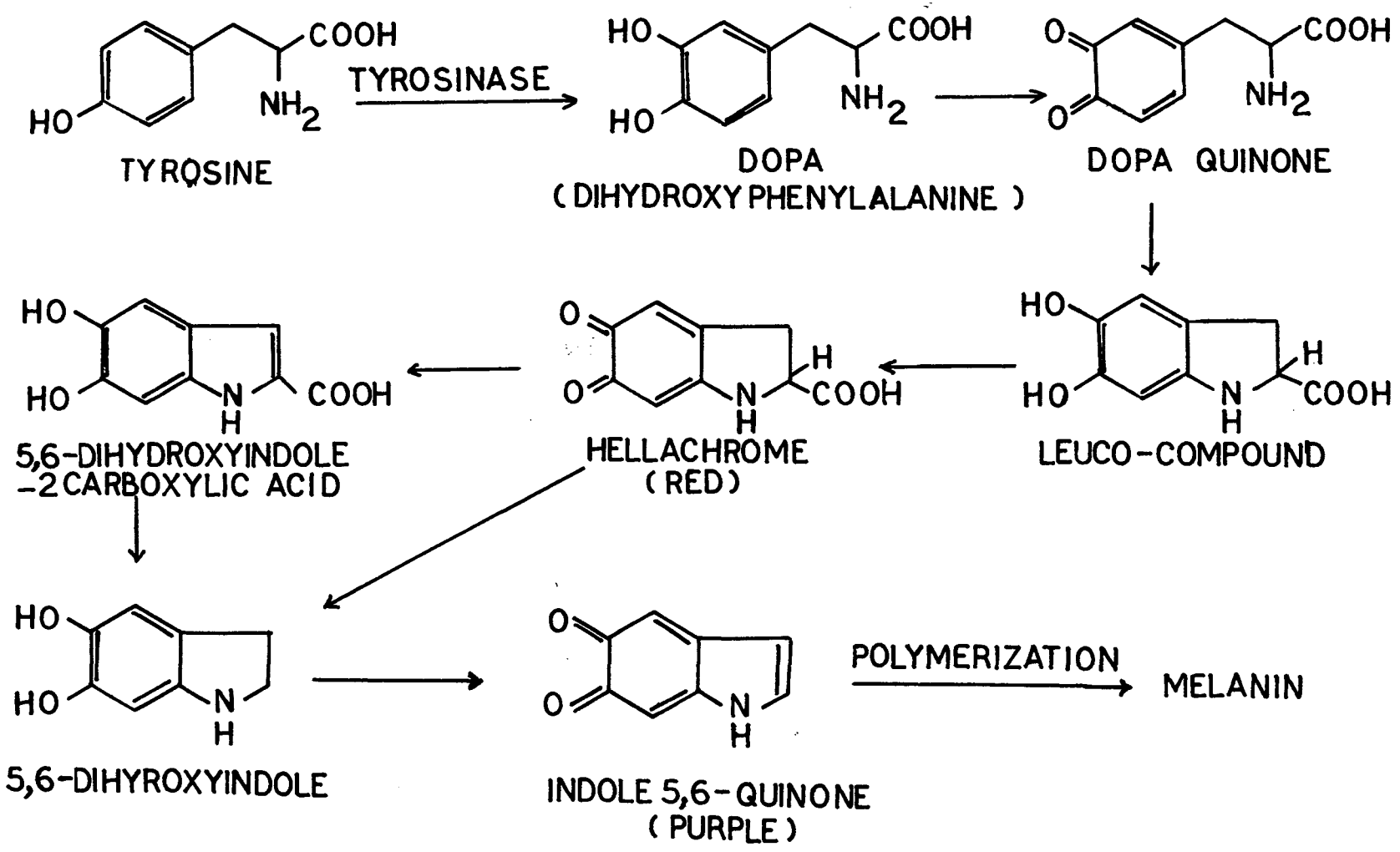
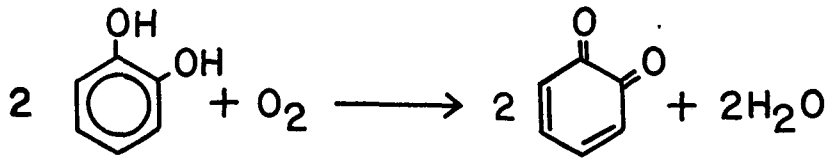


Figure 1. Synthesis of melanin from tyrosine.

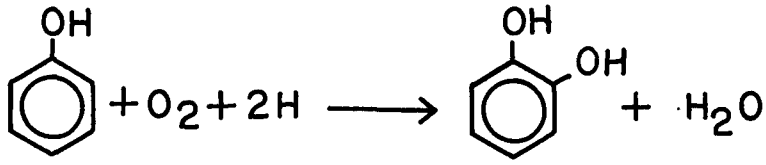
with UV light (44).

In presence of tyrosinase and molecular oxygen, tyrosine is oxidised to dopa. The reaction is usually slow at the onset, but after an induction period becomes quite fast. The conversion of tyrosine to dopa is not a reversible reaction. Dopa is oxidised enzymically by a reversible reaction to dopa- quinone. Further stages of the reaction proceed rapidly in the absence of enzyme, although the reaction rate increased in its presence. Dopa-quinone undergoes a spontaneous irreversible and rapid intermolecular change in which the nitrogen of the side chain attaches itself to the 6-position of the benzene nucleus with the formation of 5,6-dihydroxy indole-2-carboxylic acid (leuco-dopachrome). Leuco-dopachrome is readily oxidised by a reversible reaction to the corresponding quinone (dopachrome). Dopachrome is a red substance (λmax=305 - and 480 nm) and it is the first visible product formed in the reaction. Under physiological conditions dopachrome decarboxylates and undergoes a rearrangement to form 5,6-dihydroxyindole (λmax=275 - and 298 nm). The indole compound oxidized enzymically or non-enzymically to the corresponding quinone. The indole 5,6-quinone then polymerizes to melanin through polyindole quinone (λmax=305 - and 540 nm) with the consumption of approximately one atom of oxygen (15,35,76). Relatively little is known of the mechanism of this polymerization. Dressler and Dawson (35) suggested, from their studies on tyrosinase using radioactive copper, that the oxidation of monohydric phenols by tyrosinase may not involve O-dihydric phenol intermediate, rather the monophenol might be directly converted to the corresponding O-benzoquinone.

Tyrosinase is a copper-protein complex of wide distribution occurring throughout the phylogenetic scale. The enzyme isolated from the different sources shows qualitative differences. Mammalian tyrosinase is specific to tyrosine and L-(3,4-dihydroxyphenyl) alanine (dopa). Tyrosinase catalyzes the two types of reactions shown in Reaction 1 and 2. Catalysis of Reaction 1 is referred to catecholase activity of the enzyme and that of Reaction 2, the cresolase activity, in accordance with two substrates commonly employed.



(1)



(2)

The ability of enzyme to catalyze two reactions so different from each other is an interesting property and has stimulated much research. Mason et al. (1955) using O^{18} have established that oxygen of the second hydroxyl group is derived from molecular oxygen (73). The tyrosine-tyrosinase reaction is often marked by a time lag before oxidation commences (65). The induction period could be shortened by the addition of dopa (67) and there is no induction period if latter is the substrate.

Although it is rather strange from an enzymological point of

view, a one enzyme hypothesis of tyrosinase and dopa oxidase activities is now generally accepted for the following reasons:

1. Both cresolase and catecholase activities always occur together and are associated with the same electrophoretic and centrifugal components.

2. In any given preparation of the enzyme, both the activities are inhibited by same inhibitors and both activities are proportional to the content of copper and are lost when the copper is removed.

In 1957, Mason (75) proposed a scheme based on the mechanism of action of tyrosinase in which the single enzyme is able to catalyze both mono-and diphenols (Figure 2). However, Mason's scheme was rejected because it was unable to accomodate all the experimental facts, e.g., activation of enzyme by the reduction of Cu^{2+} is not possible, as the metal is now known to be present as Cu^{+}. Dressler and Dawson (35) have revealed that there might be two distinct active sites in tyrosinase molecule - Catecholase and Cresolase activity centres. Kinetic studies on tyrosinase by Shimao (137) have suggested that on the tyrosinase molecule there are two sites. One where dopa is oxidized and another where tyrosine is bound but not oxidized during the first phase of the reaction. Pomerantz (116) explained on the basis of kinetic studies that the mutual inhibition of dopa and tyrosine in the tyrosinase reaction might be present.

Tyrosinase is inhibited **in vitro** by a large number of chemicals. Such inhibition can usually be achieved by binding or removing the copper ions necessary for tyrosinase action. Common inhibitors of this

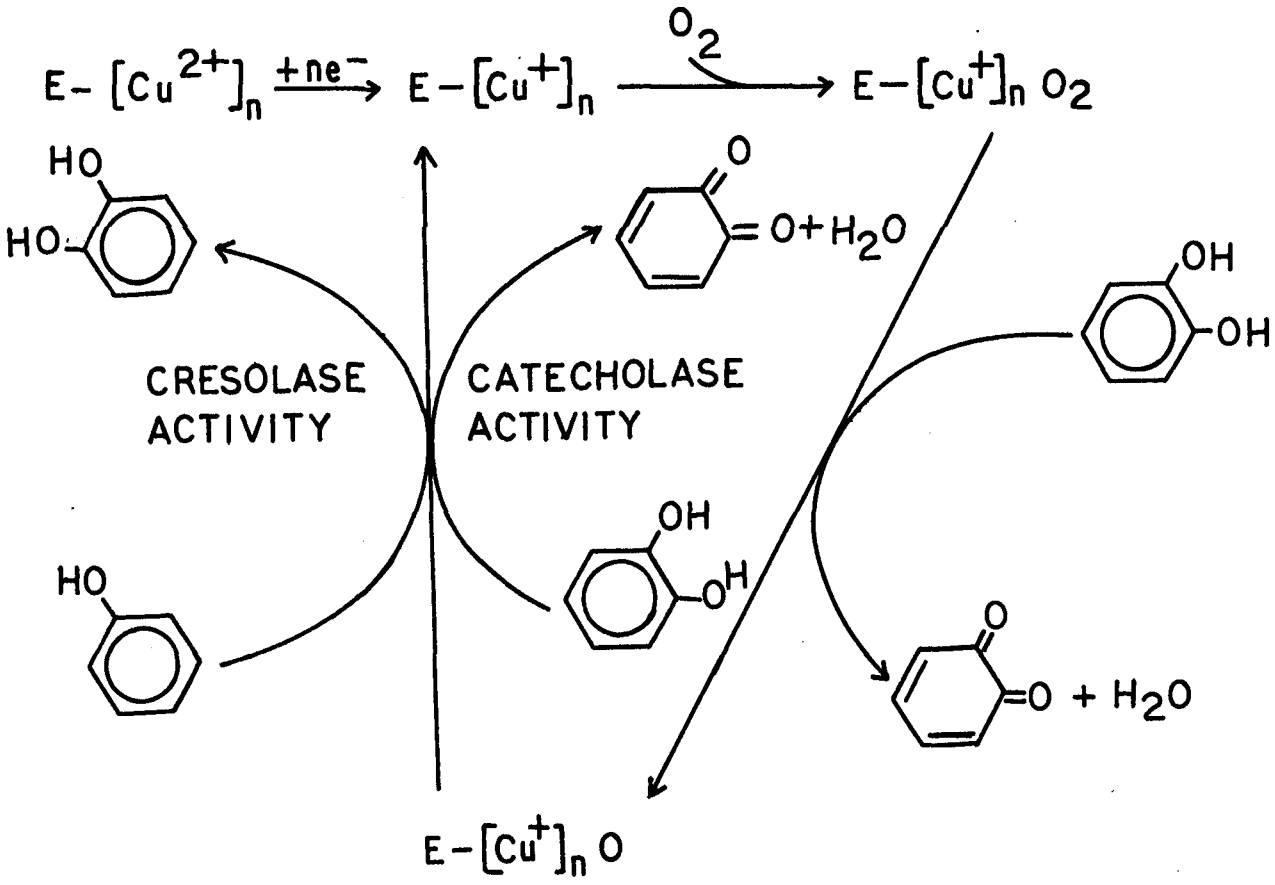


Figure 2. Mechanism of tyrosinase action according to Mason (75).

type are usually organic sulphur compounds (12,36,103), hydrogen sulphide, carbon monoxide and cyanide ions. Some of the organic sulphur compounds containing reactive sulphhydryl groups are diethyldithiocarbamate, cysteine, glutathione, 2,3-dithiopropanol (BAL), thiourea and its derivatives such as phenylthiourea, ∝-napthylthiourea and thiouracil. Carbon monoxide and cyanide ions form strong covalent bonds with copper and thereby bring about the inhibition of tyrosine - tyrosinase activity. Substrates which compete with tyrosine like 3-fluorotyrosine, N-acetyl tyrosine, N-formyl tyrosine and phenylalanine and other derivatives have been studied extensively and are of physiologically greater importance (72).

Increased pigmentation is frequently observed when heavy metals such as arsenic, bismuth, iron, gold, silver and mercury are deposited in the skin and most probably these metals bind the epidermal -SH groups thereby activating tyrosinase (99). If large amounts of these metals are present, they are capable of replacing the copper of tyrosinase giving rise to inactive enzyme and consequently to depigmentation.

Hyperpigmentation of the skin of mamals following exposure to X-rays and UV irradiations has been observed (83). Histochemically the irradiated skin shows an increased amount of melanin and a positive Dopa oxidase reaction in the epidermal melanocytes (119). However, the mechanism which regulates this radiation induced melanogenesis is not clearly known. However, it is now well understood that the process of melanin pigmentation involves the production, transfer and distribution of melanosomes. Solar or UV irradiations influence these processes.

Published reports regarding the increased melanin pigmentation following UV irradiation suggest that a numerical increase in functioning melanocytes might be responsible for enhanced pigment production (10,11,13,14,42,81,102,111,117,120,121,128,129). The existence of melanocytes which are both "enzymically" inactive (they exhibit minimal or no tyrosinase activity) and structurally unrecognizable (they do not synthesise melanosomes) have repeatedly mentioned by several investigators. These, so called dormant melanocytes are presumed to be activated as a result of exposure to UV irradiation (81).

In 1969, Lazabo et al. (130) observed that UV irradiation has a stimulating effect on the rate of melanosome formation in melanocytes and on the rate of melanization of melanosomes. In all the races (Caucasian, Negro , American, Indian and Mangol) these workers found that the number of melanosomes inside the malpighian cells become much higher after irradiation.

It is often said that UV irradiation of skin causes an increase in tyrosinase activity. In 1949 and 1950, Fitzpatrick et al. (42,43), postulated that tyrosinase in human skin exists in a partially inhibited form whereby unable to oxidise tyrosine to melanin. However, when irradiated human biopsies were incubated with tyrosine, the melanocytes were able to form new melanin. The mechanism of UV induced activation of tyrosinase as postulated by these investigators is summarised as under:

1. Oxidation of tyrosine to dopa by direct photochemical action.
2. The acceleration of tyrosine-dopa-melanin reaction by the presence of trace amounts of dopa thus formed.

Although this hypothesis is interesting but one must realise that the photochemical formation of dopa requires generally large doses of UV irradiation which under physiological conditions, seem unlikely. Hamprel et al. (48) explained the action of ultraviolet light on the basis of photochemical splitting of nucleic acid which may be responsible for erythma, an inflammatory reaction. Rothman's concept of the effect of UV irradiation is mainly the inactivation of inhibitors, cheifly -SH compounds in the skin, thereby releasing the tyrosinase and giving rise to better pigmentation. Recently Van Koert and coworkers (145-147) postulated that labilisation of melanosomal membrane by electromagnetic radiations may cause hyperpigmentation.

Further potentiation of these ultraviolet effects have been observed by in vivo administration of certain furocoumarins, e.g., psoralen and its derivatives (24, 109, 86). Ammi majus Linn (Fam. Umbelliferea) in Egypt (39) and Psoralea corylifolia Linn (Fam. Leguminosae) in India (34) were reported to be effective in bringing about pigmentation in depigmented vitiliginous sites. Since 1947, Fahmy and coworkers (38-40) isolated xanthotoxin, bergapten, imperatorin from Ammi majus. Many furocoumarin derivatives have also been synthesised (8, 16, 17, 18, 54, 55, 150). The thereapeutic use of furocoumarin in vitiligo has been originated from the work of El. Mofty et al. (37). Since then psoralen and its derivatives have been continuously used for the treatment of this disease. When applied on human skin topically and subjected to UV or solar irradiation, they cause erythma and pigment formation. Musajo et al. (86) tested the relative capacity of different coumarins in causing erythma and pigment formation on human volunteers. The compounds were dissolved in ethanol and applied

on 2-4 cm^2 size areas on the skin and were either exposed to sunlight or UV light. The erythma and pigment produced after the treatment were measured quantitatively. The results show that psoralen is most powerful of all the photosensitizing agents. Using structurally different coumarins, Pathak and coworkers (104,105,108,112) confirmed the finding of Musajo et al. (86). Musajo et al. (94) have summarized the relationship between chemical structure and skin photosensitizing activity of furocoumarins. Their finding is summarized in Figure 3. Some of the other photobiological effects produced by furocoumarins when irradiated by UV light may be summarized as (95): skin-erythma (64), death of bacteria (46,100), mutagenic effects on Drosophila melanogaster (96), inactivation of DNA viruses (89), increase in the template efficiency of DNA in RNA polymerase reaction (21) and loss of tumor transmitting capacity of Ehrlich ascites tumor cells (93).

Among the general explanations regarding the mode of action of psoralens is based on the hypothesis that the altered epidermis simply increases retention of melanin formed in ordinary amount in response to solar or UV irradiation. The second suggestion involves the stimulation of melanocytes giving rise to increased production of melanin. Judis (53) proposed the following mechanism for the action of 8-MOP.

a) It photooxidises certain inhibitors of melanin formation present in abnormal amounts in vitiliginous area.

b) It may photooxidise the available dopa to melanin.

c) It may some how raise the oxidation reduction potential in the system making conditions more favourable for normal melanin

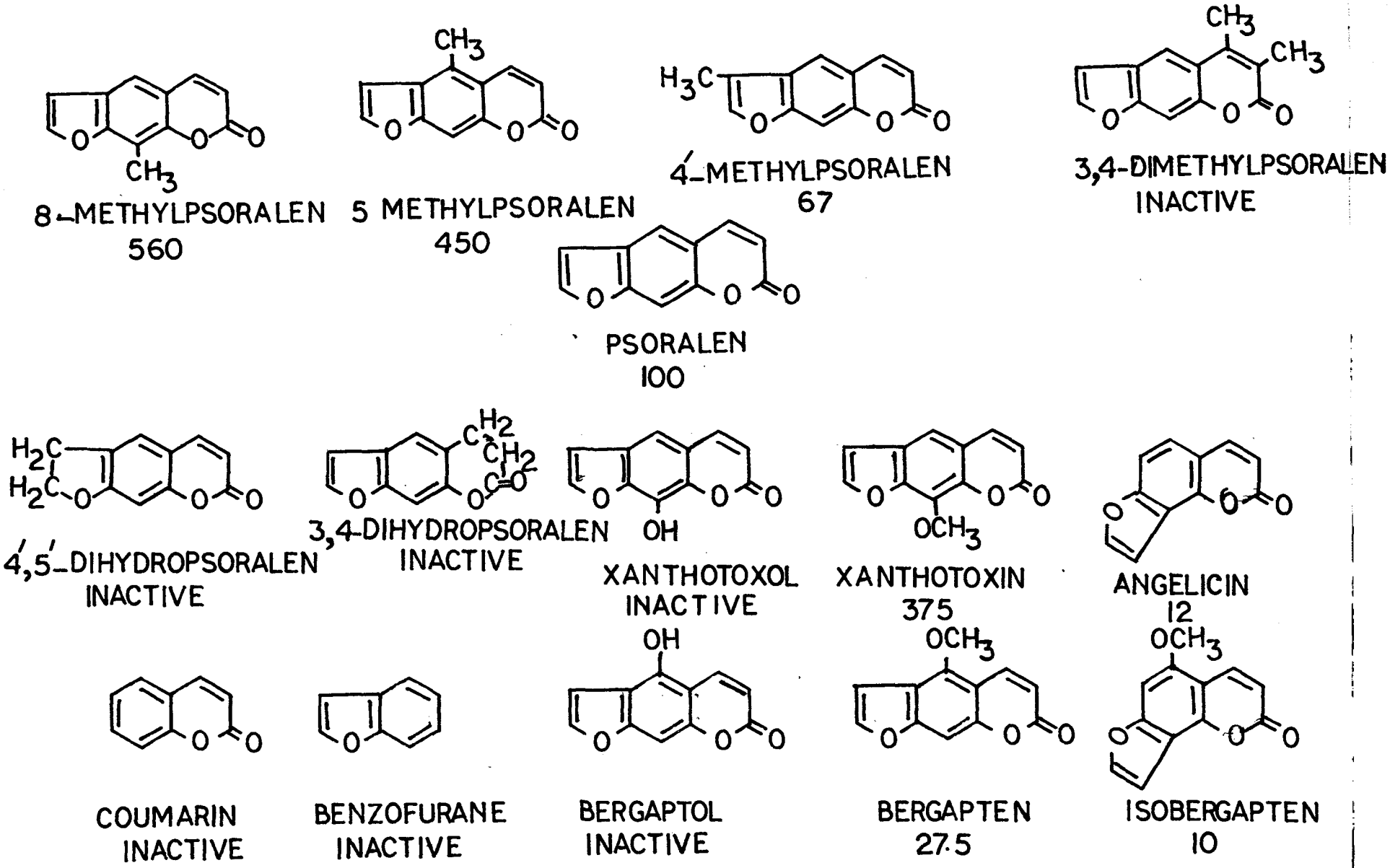


Figure 3. Relationship between structure and skin photosensitizing activity of some furocoumarins, number refers to relative activity (94).

formation.

Musajo et al. correlated the mode of action of psoralen with the photoreaction of furocoumarins with nucleic acids which has been very systematically and extensively investigated by these workers. Psoralen forms molecular complexes when added to aqueous solution of nucleic acid and irradiated by UV light. These complexes have been studied by various experimental methods such as viscosity measurements (122) equilibrium dialysis (122), spectrophotometeric measurements (27), ionic strength and furocoumarin solubilization (27) and temperature effect (27-29,89,125), all these experiments strongly supports the idea of complex formation between nucleic acid and furocoumarins. Further to clarify the reactive sites on DNA which are involved in photobinding of furocoumarins, the behaviour of the simple components of nucleic acids was examined by irradiating aqueous solutions of purine and pyrimidine bases, nucleotides and nucleosides at 365 nm in the presence of skin photosensitizing furocoumarins (87,88). The results obtained show that only pyrimidine derivatives photoreacted, forming new compounds. The structure of new compounds was elucidated after their isolation from the irradiated solution by column chromatography, on the basis of elemental analysis UV, infrared, and nuclear magnetic resonance spectroscopy (90,91). A C_4-cyclo-addition reaction of furocoumarins to the pyrimidine bases of the macromolecules takes place. Pyrimidine bases always react with their 5,6 double bond and furocoumarins may react either with 3,4 or with their 4', 5' double bond (23,41,61,62,122,123,133,148,149). It has been ascertained that in the native DNA the pyrimidine bases constitute the reactive sites, giving C_4-cyclo-addition reactions analogous to those which occur using the simple bases. In fact, photoadducts identical with those

obtained by irradiation of psoralen and pyrimidine bases have been isolated from a native DNA irradiated in the presence of psoralen and then hydrolyzed in acidic medium (92). These workers (Musajo, Rodighiero et. al.) also observed a remarkable agreement between the relative photoreactivities of different furocoumarins with native DNA and ribosomal RNA and their relative skin photosensitizing activities (22,30,31,32, 77,126). Other research workers (1-5,60,70, 106,107,110,151,152) and particularly the group of Pathak et al. have also done much of the work to investigate the mode of action of psoralen. Chakraborty et al.(19) studied, histochemically, the effect of psoralen on tyrosinase activity and melanin formation. After feeding psoralen to male toads they observed an increase in the melanin formation in skin and liver tissue.

In view of the importance of tyrosinase in melanin formation, it was considered of interest to study its interaction with psoralen. It is generally assumed that interaction between drug and a biopolymer induces a conformational change, which is responsible for the action of drug. Similar changes are also believed to occur when drug binds to unspecific receptors such as plasma proteins. Numerous methods are currently in use for studying drug protein interactions (80). However, little information is available concerning the precise nature of interaction or identity of the binding site. Fluorescence quenching has been employed to investigate conformational changes occuring in DNA as a result of binding of carcinogens and in antigen-antibody binding studies. Fluorescence quenching have been utilized here to examine the interaction occurring between psoralen and tyrosinase. The results on some aspects of the molecular morphology and the conformational stability of the native and irradiated tyrosinase in presence and absence of psoralen has also been described.

II. EXPERIMENTAL

A. Materials

1. Proteins

Mushroom Tyrosinase (lyophilized, salt free, grade III, electrophoretically pure) and Trypsin (from Bovine Pancreas type III, 2 x crystallized) were purchased from Sigma Chemical Co., U.S.A.

2. Psoralen

Gift from Central Drug Research Institute, Lucknow. The sample was chromatographically pure.

3. Substrates and Inhibitors

L-Tyrosine, E. Merck (Germany); 3-(3-4-Dihydroxyphenyl) - DL-alanine, British Drug Houses (England) and catechol, Calbiochem (U.S.A.). All these chemicals were chromatographically pure. Glutathione reduced, ascorbic acid, ethylenediamine tetraacetic acid and diethyldithiocarbamate was from British Drug Houses (England); 2-mercaptoethanol was from Calbiochem (U.S.A.); L-cysteine hydrochloride and sodium azide were

from Riedel (Germany) and 2-thiouracil was from Koch-light Laboratories Ltd. (England). All these chemicals were of analytical grade.

4. Gels

Sephadex G-200 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Sweden). Agar-agar powder used for immunodiffusion was obtained from Koiwa agar-agar Co. Ltd. (Japan) or from Difco Laboratories (U.S.A.).

5. Reagents for Polyacrylamide Gel Electrophoresis

The following reagents with their sources in parenthesis, were used in polyacrylamide gel electrophoresis.

Acrylamide, dichlorodimethyl silane, amido schwarz (E. Merck, Germany), N,N - methylene-bisacrylamide, N,N,N',N' - tetramethylethylenediamine (Fluka, Switzerland), ammonium persulphate (Riedel, Germany), bromphenol blue (B.D.H., England).

6. Animals

Rabbits having an average weight of 1-2.0 Kg were used for immunisation. They were fed on Hindustan Lever feed ad libitum and seasonal vegetables.

7. Adjuvant

Freund's adjuvant, Calbiochem (U.S.A.) was used.

8. Other Materials

The other reagents used in the preparation of buffer and other solutions were potassium hydrogen phthalate, sodiumtetraborate, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, sodium potassium tartarate, sodium carbonate, sodium acetate, sodium chloride, copper sulphate, methanol, ninhydrin, L-proline, ethyl cellosolve and urea were chemically pure and were recrystallized wherever required. The acids used included hydrochloric acid, orthophosphoric acid, acetic acid and boric acid were of reagent grade.

All glass double distilled water was used throughout these studies.

B. Methods

1. Ultraviolet Source

A long wavelength ultraviolet lamp, Spectroline (Model B-100), maximum emission 365 nm; intensity 1680 μ watt/cm^2 at a distance of 18", obtained from Black Light Eastern Inc., U.S.A., was used as the irradiation source.

2. Irradiation Procedure

Irradiation by longwave ultraviolet light was carried out in specially devised containers (6). The temperature during irradiation was regulated with the help of thermostat. All the studies were

performed at room temperature unless otherwise mentioned. Solutions were irradiated in a set of three containers and thereafter pooled.

3. Preparation of Psoralen Solution

Saturated solution of psoralen was prepared by adding few crystals in boiling phosphate buffer (of required pH) and was allowed to boil for five minutes. The suspension was brought to room temperature and the undissolved psoralen was filtered off. This stock solution contained 5 mg of psoralen per 100 ml (110). Wherever low concentration of psoralen was required, the stock solution was diluted accordingly.

4. Determination of Protein Concentration

Protein concentration was determined by the method of Lowry et al. (71). Folin phenol reagent was prepared according to Folin and Ciocalteu (45).

5. Light Absorption Measurements

Spectrophotometric measurements in the ultraviolet range were made on Carl Zeiss VSU2-P spectrophotometer or on Beckman DK-2A ratio recording spectrophotometer. The cells used were of 1 cm path length. Light measurements in visible region were made on Bausch and Lomb Spectronic 20 or on Carl Zeiss Jena spectrocolorimeter (Spekol) using glass cuvettes of 1 cm path length.

6. Fluorescence Measurements

Fluorescence was measured by Aminco-Bowmann spectrophotofluoro-

meter using glass cuvettes of 10 mm path length and 2.0 mm slit width. All glasswares were thoroughly washed with nitrating mixture before fluorescence measurements.

7. pH Measurements

pH was measured on Elico LL-10 pH meter using an Elico glass and calomel electrodes. The instrument was standardized with standard phthalate buffer in acidic and borate buffer in basic pH range.

8. Sephadex Gel Chromatography

Sephadex G-200 was allowed to swell in 0.05 M phosphate buffer (pH 6.5, μ 0.05) on boiling water bath for 5 hours. The hydrated and degassed slurry was brought to room temperature and was poured carefully into a glass column (2.2 x 37 cm) which was mounted in a vertical position on a sturdy and vibration free support. After ten minutes the outlet of the column was opened and the flow rate was adjusted to half of the operational flow rate. As the bed size increased, the flow rate was readjusted until it became approximately double of desired flow rate. The bed was stabilized by passing at least two bed volumes of the eluent. To check the uniformity in packing and to determine the void volume (Vo) of the column, 0.2% solution of Blue Dextran 2000 was allowed to pass.

Tyrosinase solution (10 mg) in phosphate buffer pH 6.5, ionic strength 0.18, was applied on the column and eluted with the same buffer at a flow rate of 15 ml per hour in 3 ml fractions. Various fractions

were tested for protein and enzyme activity. The G-200 chromatographic pattern is shown in Figure 4. Since enzyme activity corresponds to protein peak, the homogeniety of preparation on the basis of molecular size is obvious.

9. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of the enzyme was performed at pH 8.3 according to Davis (33). Siliconized gel tubes (0.5 x 7.0 cm) were held vertically with their lower ends closed by rubber stoppers. Approximately 2.5 ml of small pore gel was poured into each tube. After 30 minutes of polymerization, nearly 0.2 ml large pore gel, containing approximately 100-200 µg of protein, was layered on to the top and photopolymerized for about 40 minutes. A current of about 5 mA per tube was maintained during electrophoresis. The buffer in the upper chamber contained bromophenol blue as tracking dye.

(a). Development of Activity Bands and Protein

After the electrophoresis the gels were removed and were stained for protein and activity bands. Protein stained with amido schwartz (1%) in 7% acetic acid for 10 minutes. The background stain was removed by successive rinses with 7% acetic acid. For the activity bands, the gels were flooded with the substrate solution (DL-Dopa, 1.5×10^{-3} M in 80% ethyl alcohol) and left until colour band developed. Ethyl alcohol 80%, facilitated the development of bands (25).

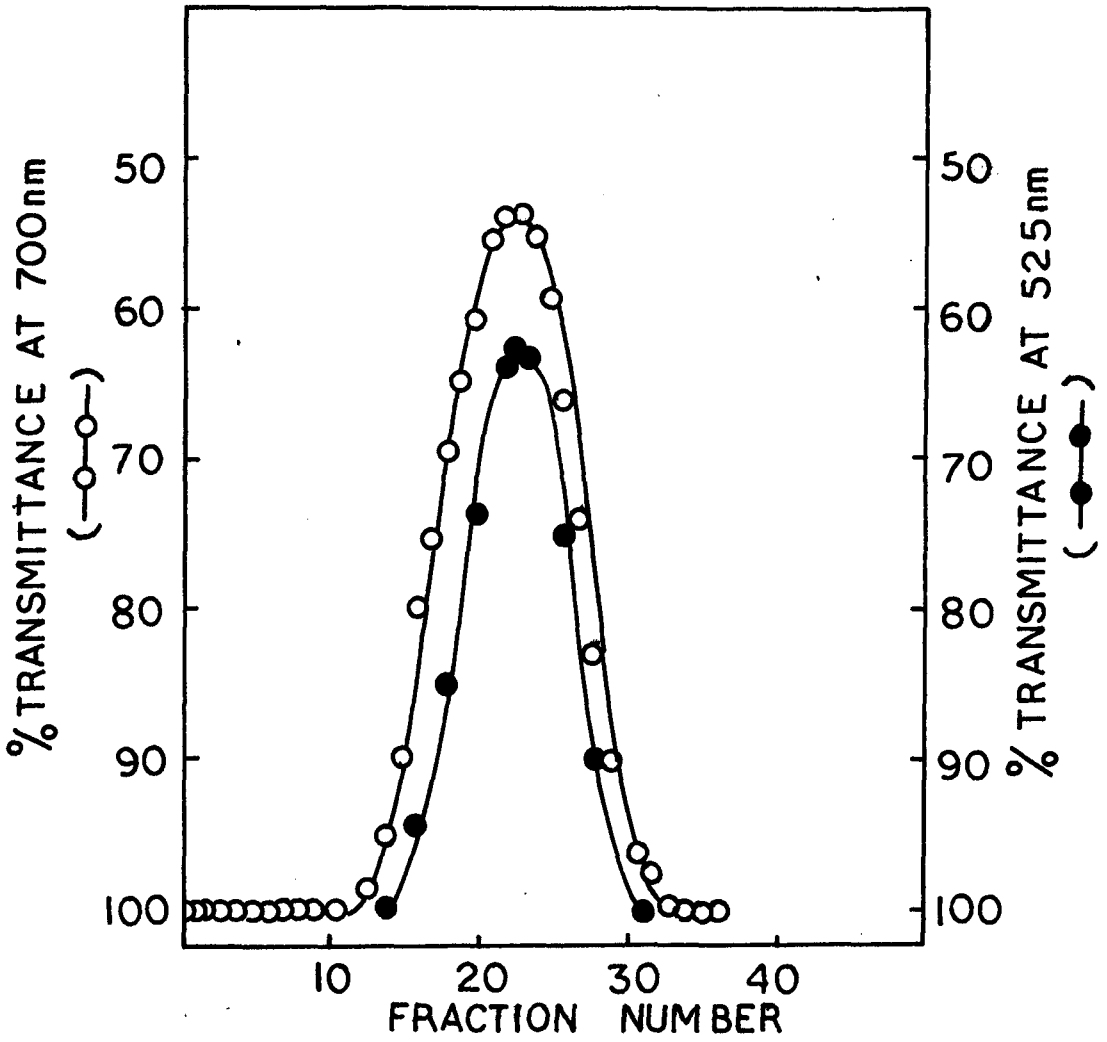


Figure 4. Chromatographic pattern of mushroom tyrosinase on Sephadex G-200. 10.0 mg of protein was applied on the column (2.2 x 37 cm); flow rate 15 ml per hour; fraction size 3.0 ml; (—o—) per cent transmittance at 700 nm; (—●—) per cent transmittance at 525 nm.

10. Immunization and Collection of Antisera

(a). Preparation of the Antigen Solution and Immunization

Rabbits were immunized using native tyrosinase emulsified with Freund's complete adjuvant (one volume of adjuvant plus two volumes of protein). Twenty milligram protein in 1.5 ml emulsion was injected in rabbits at multiple sites. Each rabbit received a total of 30 mg of tyrosinase.

After a rest of 20 days, blood was taken out two times on alternate days by cardiac puncture. Approximately 5-10 ml of blood was withdrawn each time.

(b). Preparation of Antisera

After keeping the blood in clean, dry centrifuge tubes at 37° for about two hours, serum was separated with the help of clean, dry, thin, round tipped glass rod in order to avoid the hemolysis of red blood cells and centrifuged for half an hour at 2000 - 3000 rpm. The supernatant was carefully decanted and kept in a clean, dry container.

11. Detection of Antibody

The following tests were performed to detect the presence of antibody in the serum:

(a). Ring Test:

Approximately 0.3 ml of antiserum was taken in each of the six

microtubes which were then overlaid with 0.3 ml tyrosinase solution of increasing dilution. The following controls were prepared. First tube contained antigen and normal serum. In second tube normal serum was replaced by normal saline and in third tube antiserum and normal saline were taken. After incubation at 37° for few hours, a positive test was indicated by the presence of precipitate at the junction of two liquids.

(b). Precipitin Test in Agar Gel

Immunodiffusion was performed according to Ouchterlony's technique. One per cent agar in normal saline was poured on petridishes and central and peripheral wells were made. The antibody was applied to the central well and the antigen was placed in the peripheral wells. The petri dishes were kept at 37° for 1 hour and then for 2-3 days at 4°. Precipitin arcs were located by proper staining with 1% amido schwartz and by substrate staining.

12. Tryptic Hydrolysis

Tryptic digestion of native and irradiated tyrosinase was performed at pH 7.5 and 33° according to Paik and Kim (101) and the extent of digestion was estimated by ninhydrin reaction. The reaction mixture was prepared in 0.1M sodium phosphate buffer, pH 7.5, by mixing 1.5 ml of the protein (750 µg) with 1.5 ml of trypsin (750 µg) at 33°. Aliquots of 0.5 ml were taken from the reaction mixture at different time intervals, mixed with 0.5 ml sodium acetate buffer pH 5.1, pre-cooled in an ice bath, to stop the reaction. After the addition of ninhydrin reagent (0.5 ml) the mixture was heated on a boiling water bath for 15 minutes and then cooled in cold water for few minutes.

Ten milliliters of 50% (V/V) ethanol was added to dilute the colour. The precipitate was filtered off through Whatman No.1 paper and absorbancy recorded at 570 nm.

13. Ninhydrin Reaction

Ninhydrin reaction was carried out by the method essentially due to Moore and Stein (84). The reagent was prepared by dissolving 2.0 g of ninhydrin, 0.3 g of hydrindantin in 75 ml of methyl cellosolve followed by the addition of 25 ml of 4 M sodium acetate buffer pH 5.5.

14. Activity Measurements

(a). L-Tyrosine and DL-Dopa as Substrate

Enzyme activity with tyrosine and dopa as substrates was determined by the method of Horowitz (51). The reaction mixture contained 1.0 ml of 0.002 M L-Tyrosine or 0.002 M 3-(3,4-Dihydroxyphenyl) DL-alanine solution, 2.0 ml of 0.1 M phosphate buffer (pH 6.5) and 1.0 ml enzyme solution. After incubation for 15 minutes at 30°, the colour intensity of dopachrome formed was measured at 480 nm.

(b). Catechol as Substrate

Method of Jolly and Mason (52) was used for determining the enzyme activity with catechol as the substrate. Enzyme activity was measured by determining the amount of quinone formed. Quinone, the oxidised product of catechol, reacts with proline to produce a red compound. The colour thus formed is stable at pH 6-7, which is the

range of optimum enzyme activity. By the use of this reaction the enzyme activity at 30° was determined at 525 nm. To 2.0 ml of substrate, equimolar mixture of 0.01 M catechol and 0.01 M proline, was added followed by 0.5 ml of enzyme and 1.5 ml of buffer. The mixture was mixed quickly and read at 525 nm after different known intervals.

III. RESULTS

I. INTERACTION BETWEEN PSORALEN AND TYROSINASE IN ABSENCE OF ULTRA VIOLET LIGHT.

1. Fluorescence Spectra of Tyrosinase

Excitation and emission fluorescence spectra of tyrosinase were measured in the wavelength range 250 to 440 nm (Figure 5). The excitation and emission maxima of enzyme were at 280 nm and 350 nm respectively. These characteristic maxima are generally observed with proteins containing tyrosine and tryptophan (143, 144).

2. Fluorescence Spectra of Psoralen

Excitation and emission spectra of psoralen shown in Figure 6, was measured in wavelength range 280 to 600 nm. The excitation maximum was observed at 330 nm which is identical to one of its UV absorption maxima. The emission maximum was at 450 nm, similar to that reported by Bodighiero et al.(89).

3. Interaction of Psoralen with Tyrosinase

Decrease in fluorescence intensity of tyrosinase or psoralen at

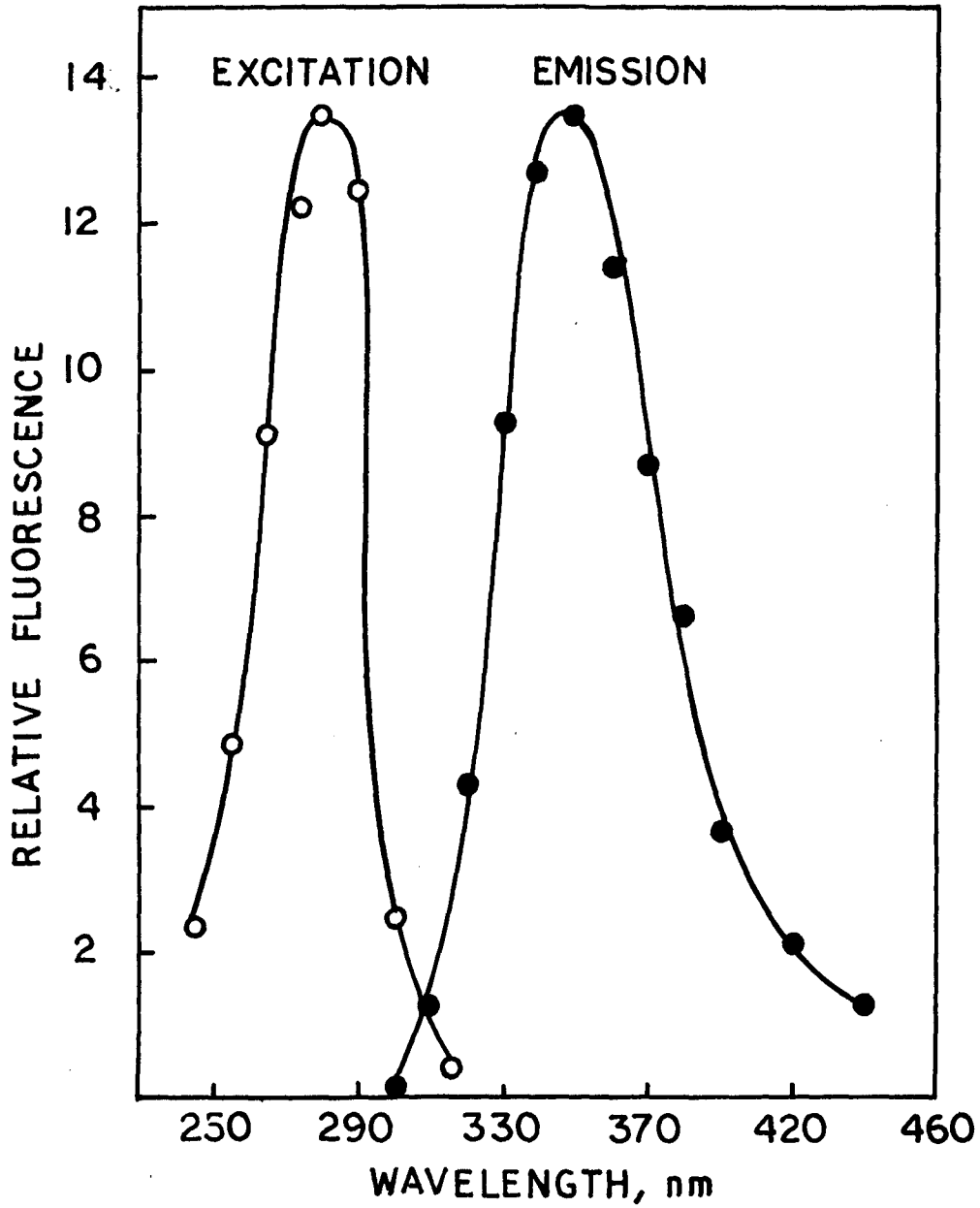


Figure 5. Fluorescence excitation and emission spectra of tyrosinase in 0.1 M sodium phosphate buffer, pH 6.5, ionic strength 0.18; enzyme concentration 0.1 mg/ml; temperature 30°. Fluorescence was corrected for solvent blanks.

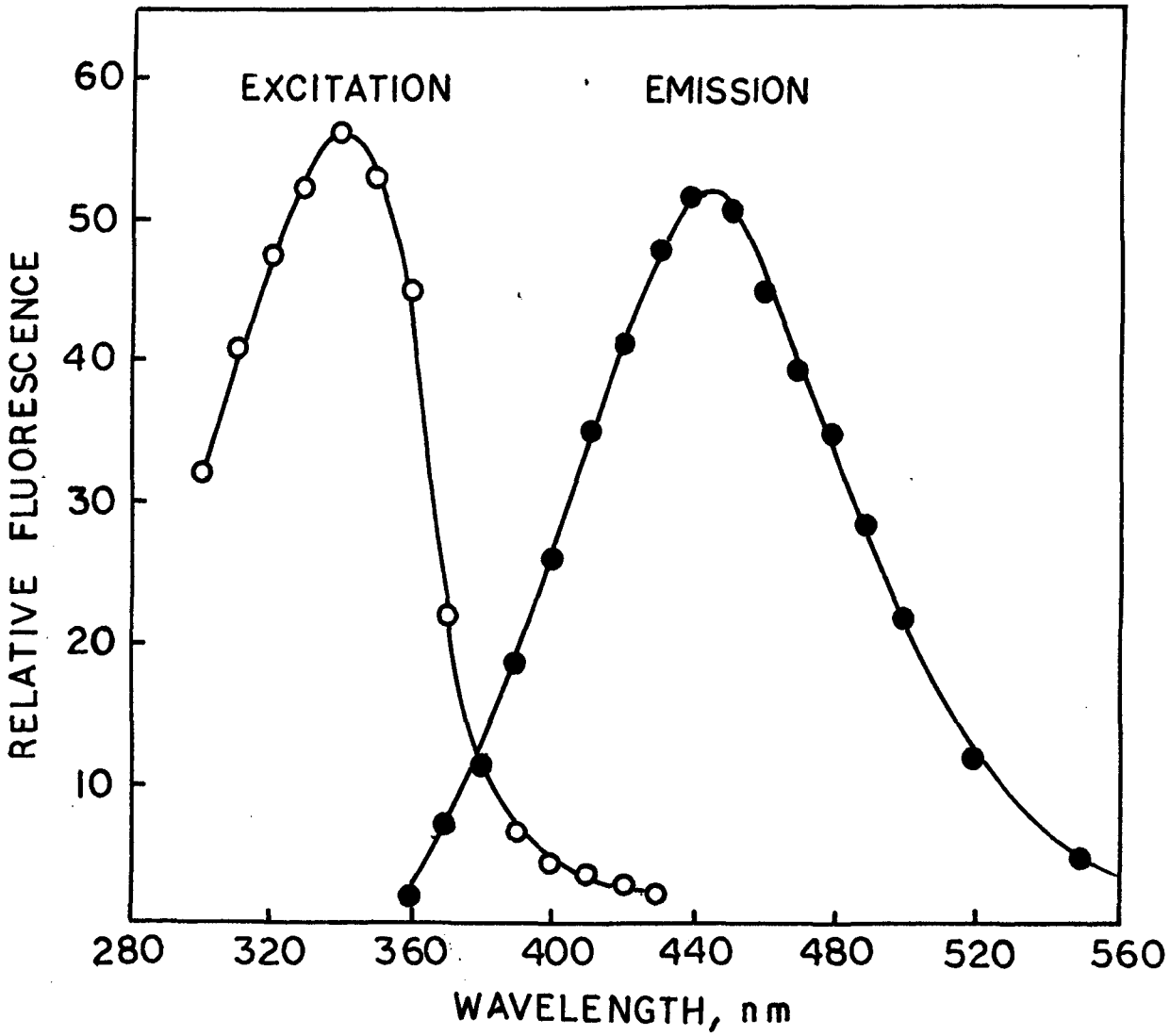


Figure 6. Fluorescence excitation and emission spectra of psoralen in 0.1 M sodium phosphate buffer, pH 6.5, ionic strength 0.16; psoralen concentration 25 µg/ml and temperature 30°. Fluorescence was corrected for solvent blanks.

their emission maxima was taken as a measure of interaction. No spectral shift was observed under our experimental conditions. The quenching of fluorescence has been used as a measure of interaction between biopolymer (DNA and protein) and small molecules (97,139). The fluorescence emission spectra of psoralen were determined in the presence of different concentrations of tyrosinase (16.0 µg/ml to 116 µg/ml) and results have been shown in Figure 7. Continuous decrease in psoralen fluorescence was observed, with increasing tyrosinase concentration. At tyrosinase concentration of 116 µg/ml, the fluorescence of psoralen is decreased to 14.6 per cent (Table I). In absence of psoralen, as the concentration of enzyme increased, a gradual increase in its fluorescence intensity was observed upto a concentration of 50 µg/ml (Figure 8). However, further increase in enzyme concentration resulted in decreased fluorescence. Marked quenching in enzyme fluorescence was observed at all concentrations, when its fluorescence intensity was measured in the presence of psoralen. At enzyme concentration of 50 µg/ml, the per cent decrease in fluorescence intensity was about 35 per cent. By using the data of Figure 8 and assuming that the decrease in relative fluorescence represents interaction of psoralen with tyrosinase, the association constant, K, was calculated by the help of Stern-Volmer equation (49,138).

$$(Fo/F) - 1 = (1 - p)\ Cb/C + pCb$$

where C and Cb are the equilibrium concentrations of free and bound psoralen, F and Fo, respectively, represent the relative fluorescence in presence and absence of enzyme and p is the ratio of quantum yields of bound and free psoralen. The slope of the straight line plot between

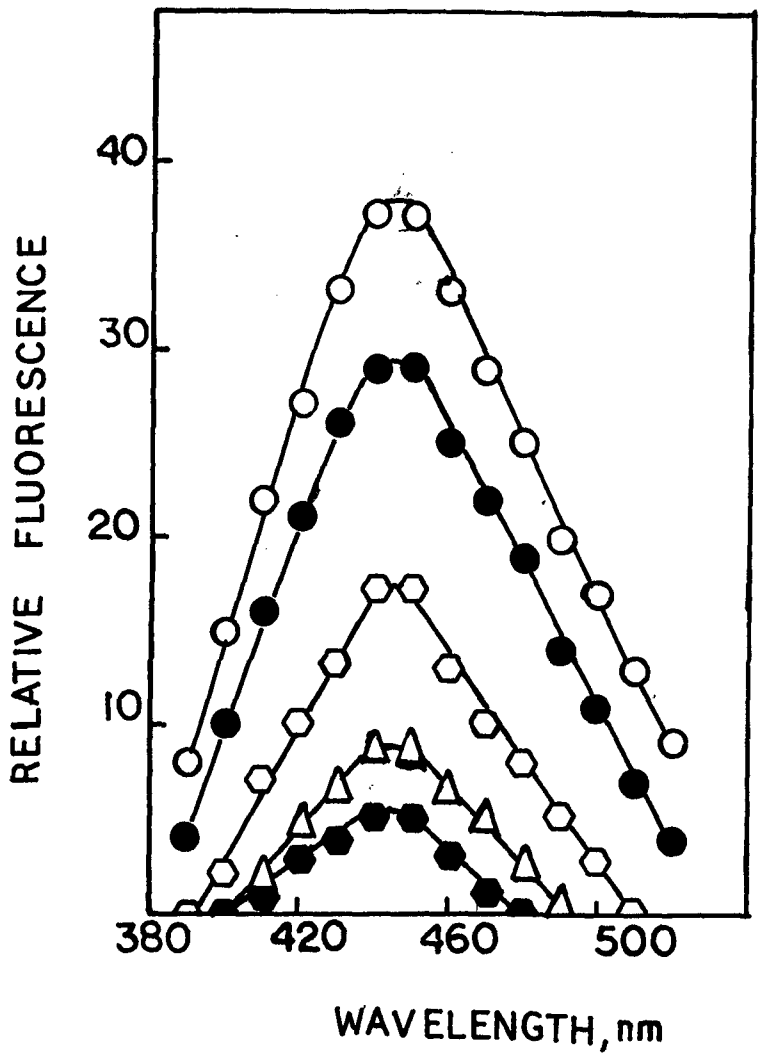


Figure 7. Fluorescence spectra of psoralen-tyrosinase system. The concentration of tyrosinase was varied from 0 to 116.6 µg/ml at a fixed concentration of psoralen (16.6 µg/ml). Concentration of tyrosinase: (—○—) zero; (—●—) 16.6 µg/ml; (—◇—) 50 µg/ml; (—△—) 83.3 µg/ml; (—◆—) 116.6 µg/ml. The wavelength of excitation was 330 nm. The solutions were prepared in 0.1 M phosphate buffer pH 6.5 at room temperature (30°).

TABLE I

EFFECT OF TYROSINASE ON FLUORESCENCE INTENSITY OF PSORALEN

Psoralen concentration 16.6 µg/ml pH 6.5

Temperature 30°

Tyrosinase (µg/ml)	Fluorescence at 450 nm (%)
0	100.0
16.6	77.3
50.0	41.3
83.3	22.6
116.6	14.6

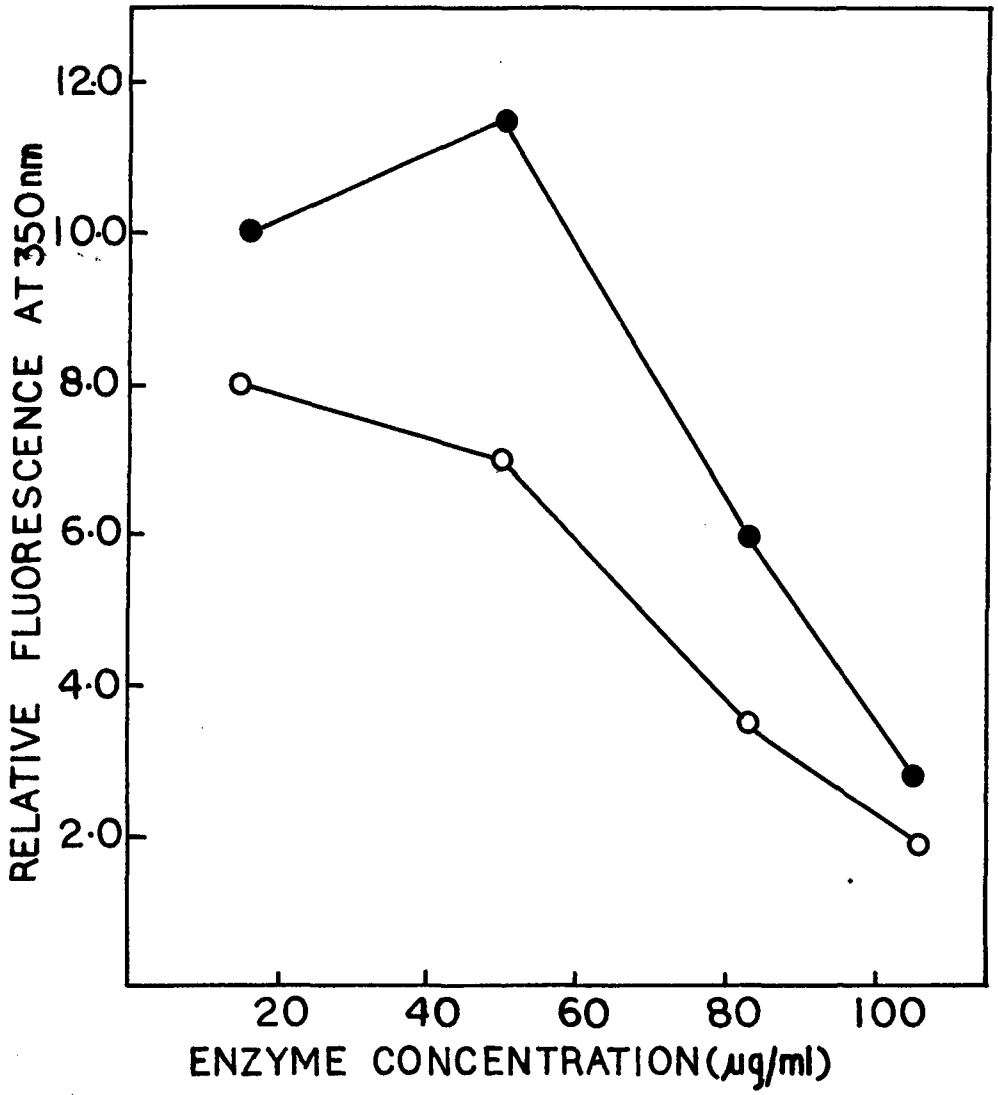


Figure 8. Relative fluorescence of tyrosinase in absence (—●—) and presence (—○—) of psoralen as a function of enzyme concentration. The concentration of tyrosinase was varied from 0 to 116 µg/ml at a fixed concentration of psoralen (16.6 µg/ml). The excitation wavelength was 280 nm and the solutions were prepared in 0.1 M phosphate buffer pH 6.5, at room temperature (30°).

(Fo/F) - 1 versus 1/Enzyme concentration determined by the method of least squares yielded the value of (1 - P) K (Figure 9A). Further, a plot of Fo/F versus 1/Enzyme was plotted (Figure 9B), a least squares analysis yielded the intercept on Fo/F axis which was equal to 1/p. Thus the equilibrium constant for the association of psoralen and tyrosinase was calculated. The value of association constant K, was found to be 8.4×10^6 M^{-1} at pH 6.5, ionic strength 0.05 and temperature 27^0. Using the value of K the free energy change was calculated by the equation:

$$\triangle F = -RT \ln K$$

where $\triangle F$ is free energy change, R and T are gas constant and absolute temperature respectively. The value of free energy change was calculated and found to be -9.0 Kcal/mole.

In order to know the effect of psoralen concentration on the interaction, the enzyme concentration was kept constant and its fluorescence intensity was observed as a function of psoralen concentration (Figure 10). As evident from the figure, the fluorescence intensity of enzyme in presence of psoralen decreased abruptly during the initial increase in psoralen concentration. The point of intersection was determined after extrapolating the curve and was found to be 16×10^{-5} M of psoralen at 7.8×10^{-7} M enzyme concentration. The molar ratio or the number of psoralen molecules interacted with one molecule of enzyme was calculated and found to be 77 at pH 6.5, which is the optimum pH as we shall see latter.

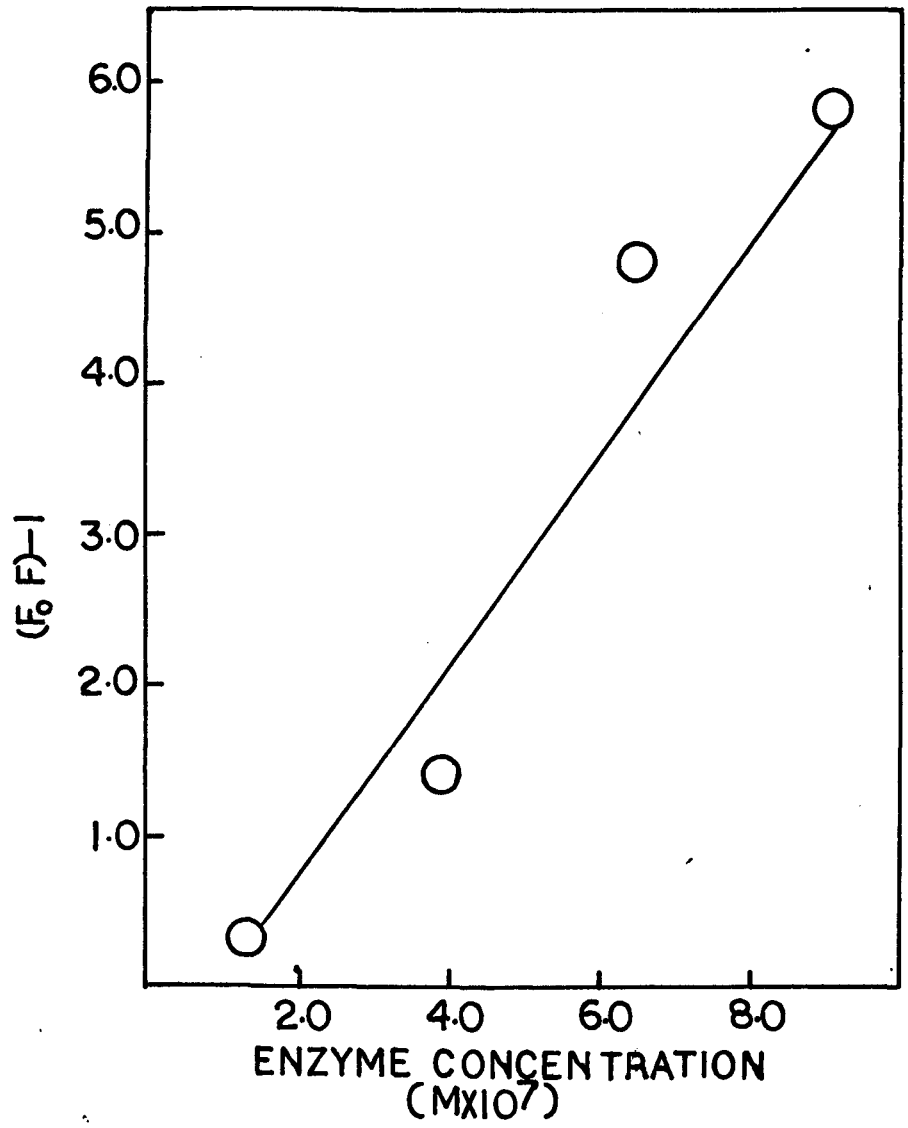


Figure 9. (A). Fluorescence quenching of tyrosinase by psoralen. Analysis of experimental points of Figure 8 was performed by the method of least squares using Stern-Volmer equation (see text).

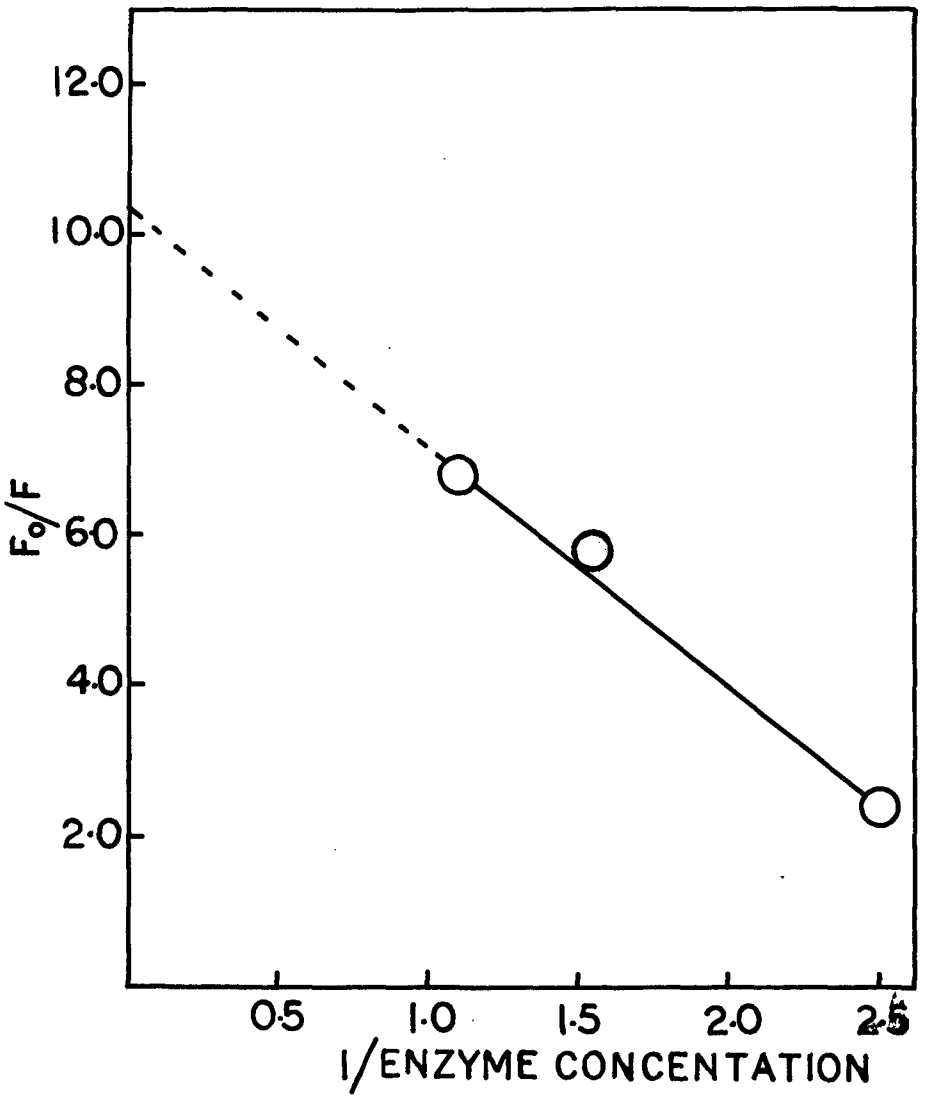


Figure 9. (B). Fo/F1 versus 1/Enzyme concentration plot for tyrosinase-psoralen system. Other experimental conditions as in Figure 8.

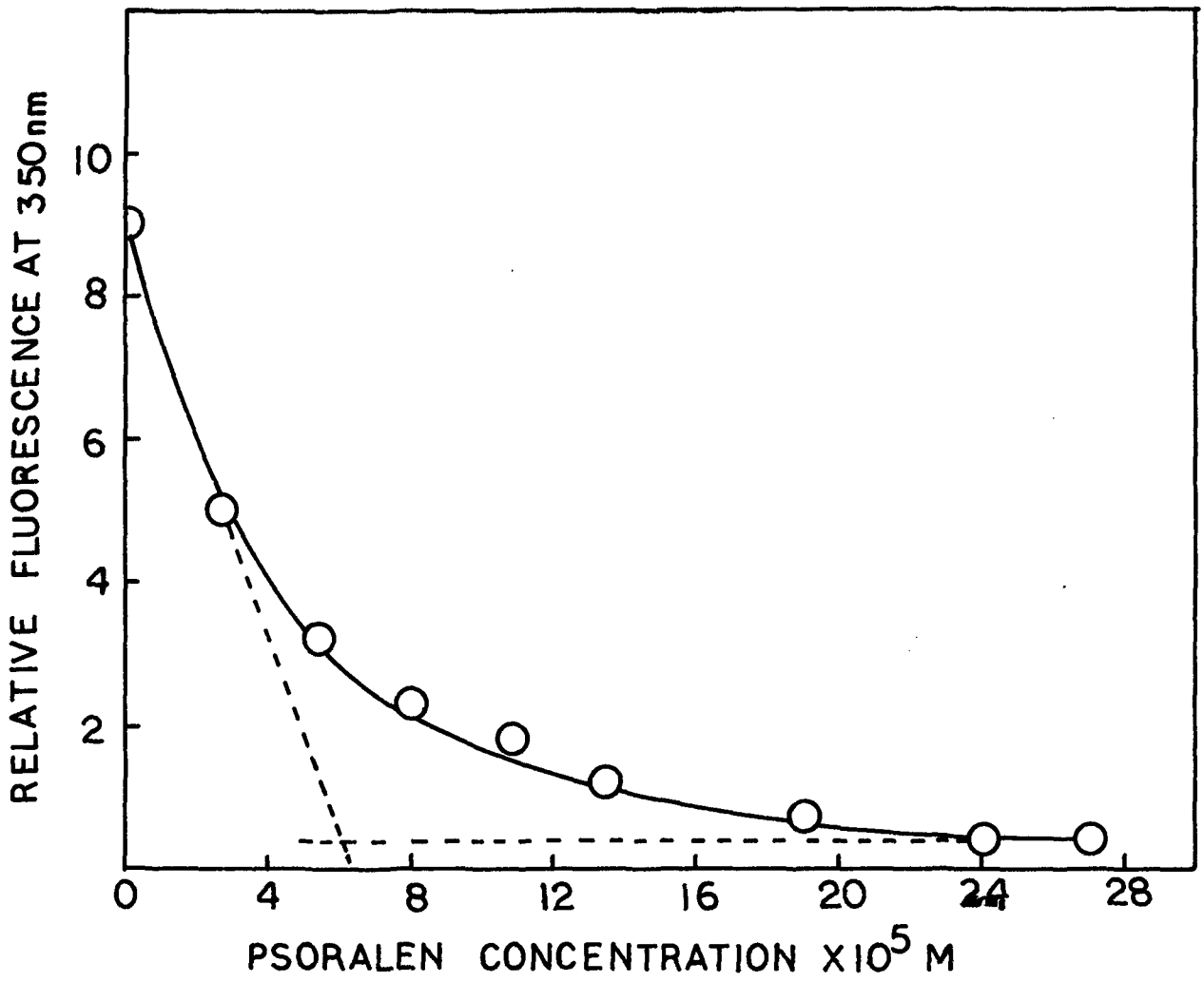


Figure 10. Fluorometeric titration of tyrosinase in 0.1 M sodium phosphate buffer (pH 6.5; ionic strength 0.18) at room temperature (30°). To a fixed enzyme concentration (200 µg/ml) was added increasing concentration of psoralen (0 - 28 x 10^{-5} M) and the fluorescence was recorded. The enzyme concentration at the point of intersection was 7.8 x 10^{-7} M. Excitation wavelength was 280 nm.

(i). Effect of pH on the Binding of Psoralen with the Enzyme

The point of intersection was determined at different pH values (3.5, 5.0, 6.5 and 8.0) using Britton-Robinson buffer (113). The calculated molar ratio at different pH are delineated in Table II. The data indicate that the molar ratio increases with increase in pH. The molar ratio of psoralen-tyrosinase system was 77 at pH 6.5 and 92 at pH 8.0.

(ii). Effect of Psoralen on Tyrosinase Activity

The dual catecholase and cresolase activities of tyrosinase was determined as a function of psoralen concentration. Per cent increase in enzyme activities in presence of psoralen (2.7×10^{-5} to 13.5×10^{-5} M/lit) are depicted in Figure 11. It is evident from the data that enzyme showed relatively higher activities (catecholase and cresolase) at pH 6.5. Increase in both the activities was observed with increasing psoralen concentration. The maximum increase in catecholase and cresolase activity was determined to be 47% and 42% respectively in presence of 13.5×10^{-5} M psoralen. No significant increase in cresolase activity was noticed at pH 5.0, however, a small but significant increase in catecholase activity was observed.

(iii). Effect of Ionic Strength

Fluorescence intensity of enzyme in presence and absence of psoralen at varying ionic strengths (0.01 to 0.1) was measured and results are shown in Figure 12. There was no effect of salt concentration on the fluorescence intensity of enzyme in presence and absence

TABLE II

EFFECT OF pH ON THE MOLAR RATIO OF TYROSINASE - PSORALEN SYSTEM

Tyrosinase concentration 7.8×10^{-7} M Ionic strength 0.05

Temperature 30°

pH	Psoralen* (10^{5}M)	Molar Ratio
3.5	4.0	51.2
5.0	5.6	71.8
6.5	6.0	77.0
8.0	2.5	32.0

* at intersection point.

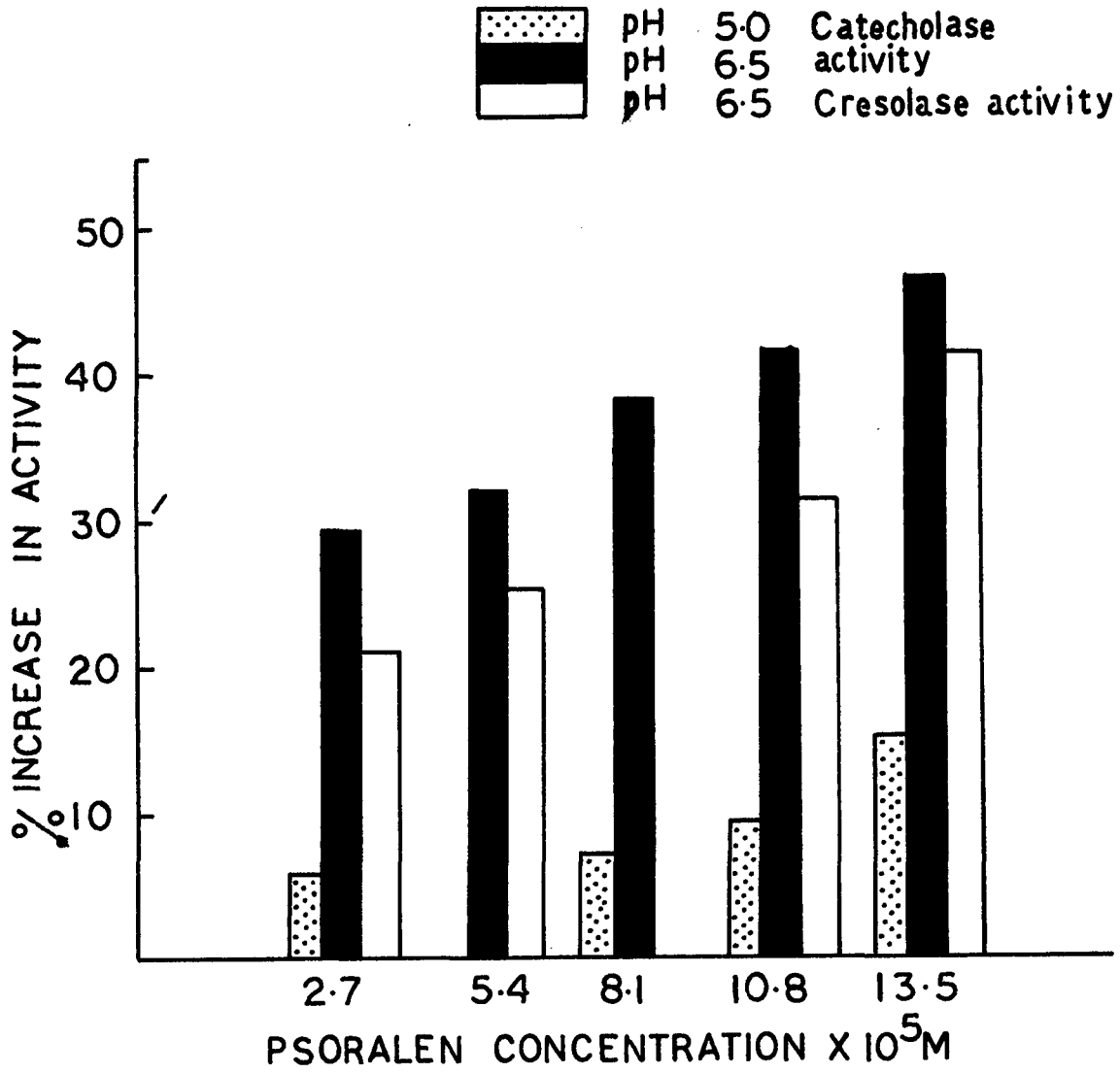


Figure 11. Effect of psoralen on tyrosinase activity. The solutions were prepared in Britton-Robinson buffer of different pH values. The concentration of psoralen was varied from 2.7 x 10^{-5} to 13.5 x 10^{-5} M. Catechol (10 mM) and tyrosine (2 mM) solutions were used for measuring catecholase and cresolase activities respectively.

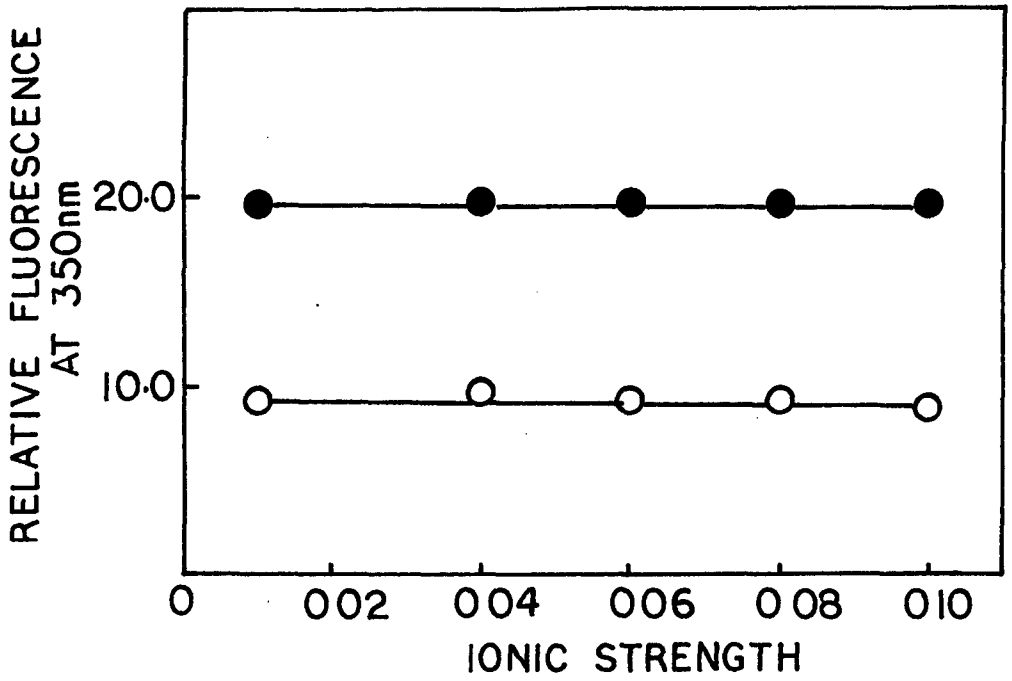


Figure 12. Effect of ionic strength on the relative fluorescence of tyrosinase in presence (—○—) and absence (—●—) of psoralen. The concentrations of tyrosinase and psoralen were 13.25 µg/ml and 12.5 µg/ml respectively. Solutions were prepared in 0.1 M sodium phosphate buffer pH 6.5; sodium chloride was used for maintaining ionic strength; the wavelength of excitation was 280 nm.

of psoralen. However, a marked constant drop in fluorescence at all ionic strengths suggests an interaction between psoralen and enzyme.

The enzyme activity was also measured under similar conditions and results are shown in Figure 13. It is clear from the activity data that the activity of enzyme was independent of the salt concentration, in presence and absence of psoralen.

(iv). Effect of Denaturant

Fluorescence intensity of enzyme at different concentrations of urea (0 - 8 M) was measured in presence and absence of psoralen (Figure 14). The relative fluorescence of enzyme increases with increasing urea concentration (Figure 14 a). About 52 per cent increase in fluorescence was observed at 8M urea. However, in presence of psoralen, the fluorescence intensity increases with increasing urea concentration but extent of increase was comparatively less, (Figure 14 b). In this case increase in fluorescence at 8M urea was only 27 per cent, which is about half of enzyme without psoralen.

The biological activity of enzyme is also decreased with increasing denaturant concentration and activity is completely lost in 8M urea. In presence of psoralen, the biological activity of enzyme is also decreased, but the extent of decrease is appreciably less than that without psoralen (See Table III).

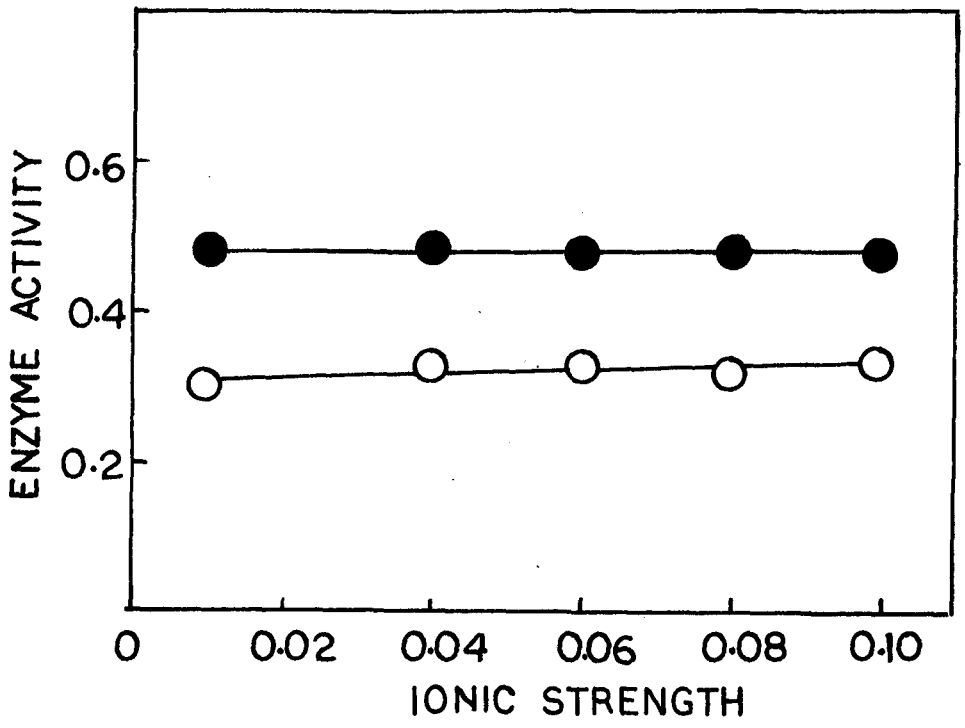


Figure 13. Effect of ionic strength on tyrosinase activity in presence (—○—) and absence (—●—) of psoralen. Activity was measured using 2 mM tyrosine as substrate at 30°. Other experimental conditions were similar to that of Figure 12.

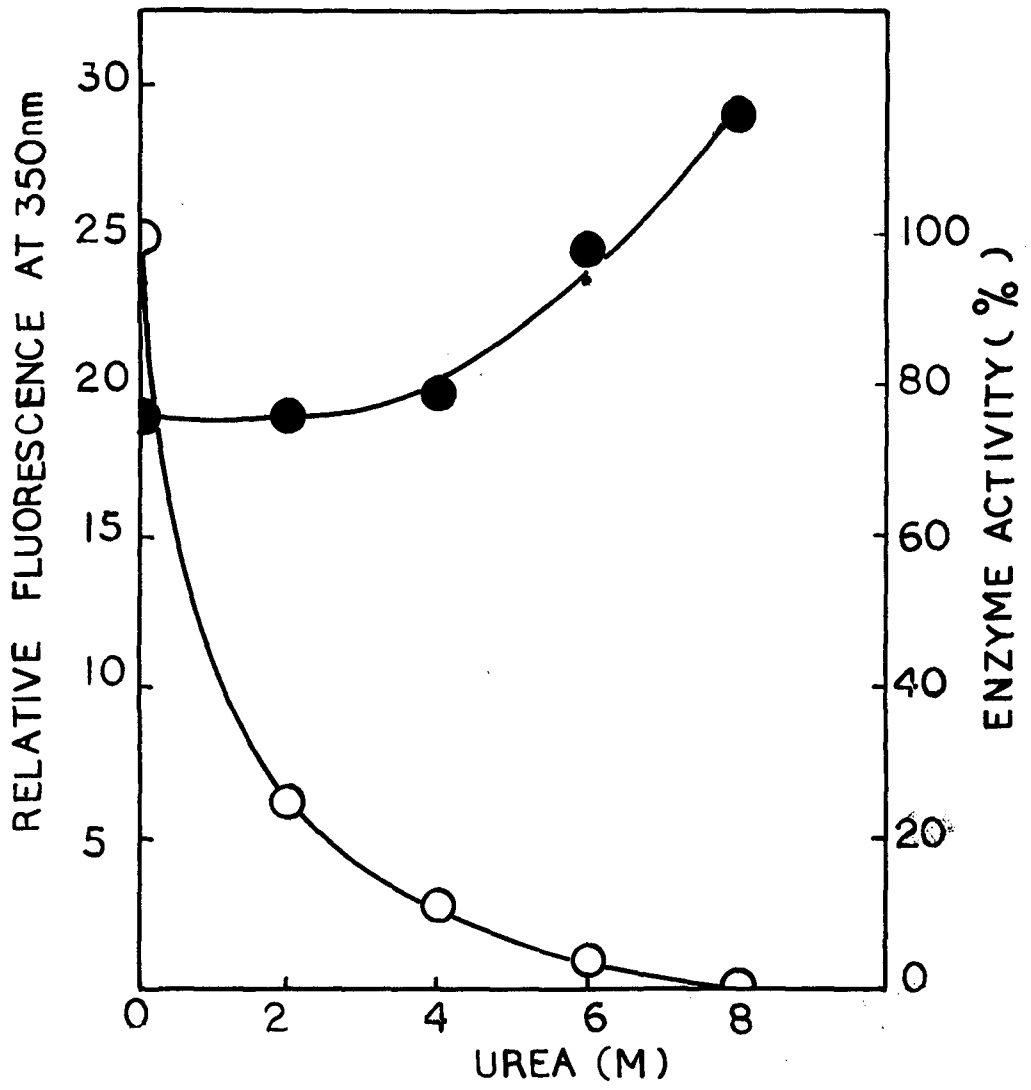


Figure 14. (A). Effect of urea on the relative fluorescence (—●—) and biological activity (—○—) of tyrosinase. The concentration of tyrosinase was 100 µg/ml. Solutions were prepared in 0.1 M phosphate buffer pH 6.5; the wavelength of excitation was 280 nm; 2 mM Dopa was used as substrate for measuring the enzyme activity.

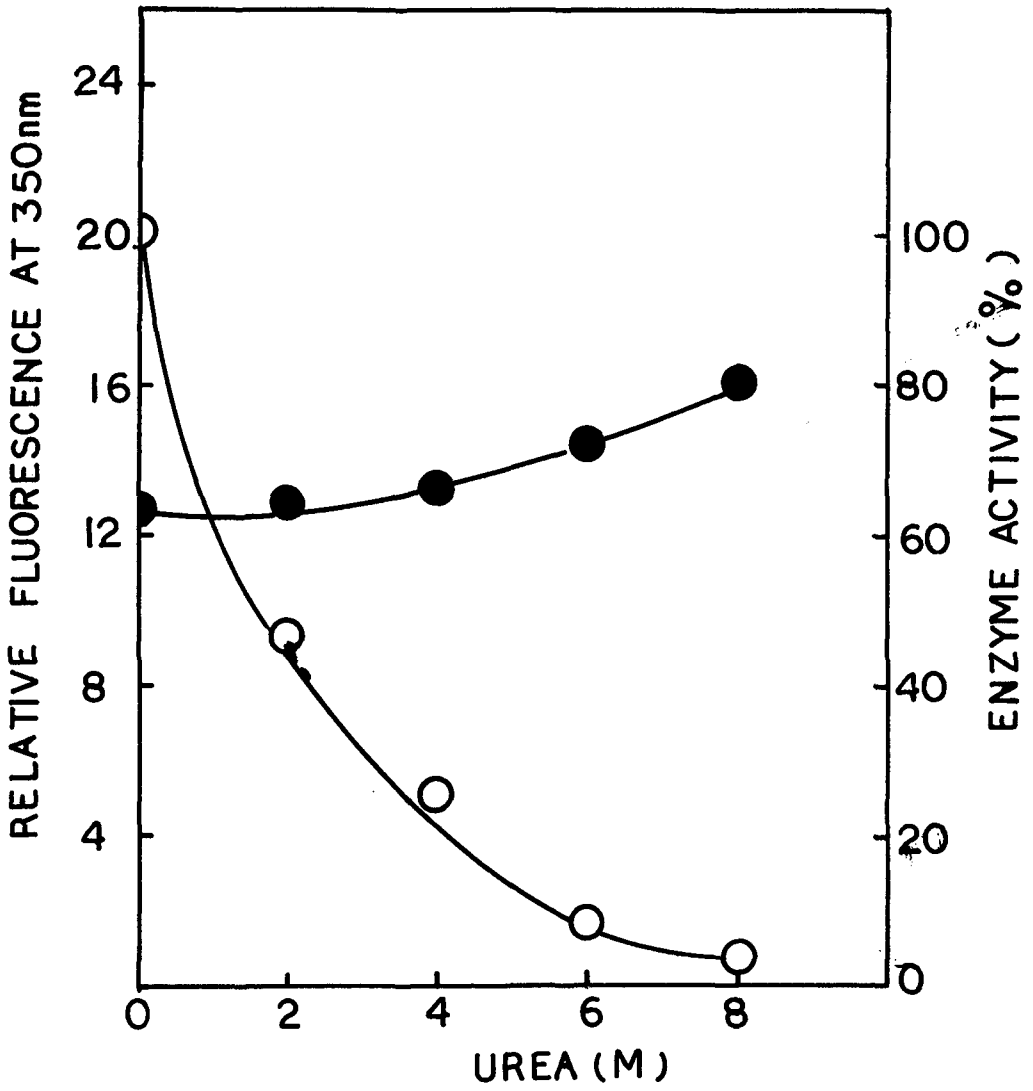


Figure 14. (B). Effect of urea on relative fluorescence (—●—) and (—○—) biological activity of tyrosinase in presence of psoralen. The concentration of tyrosinase and psoralen were 100 µg/ml and 5 µg/ml respectively. Other details as in Figure 14 (A).

TABLE III

EFFECT OF UREA ON TYROSINASE ACTIVITY

Enzyme concentration 25 µg/ml. pH 6.5

Psoralen concentration 5 µg/ml. Ionic strength 0.18

Urea (M)	Enzyme Activity* (%)	
	- Psoralen	+ Psoralen
2	25.2	46.2
4	11.0	25.2
6	4.0	8.3
8	0	4.3

* Enzyme activity was measured using DL-Dopa (2 mM) as substrate.

II. INTERACTION BETWEEN PSORALEN AND TYROSINASE IN THE PRESENCE OF ULTRAVIOLET LIGHT.

1. Fluorescence Spectra of Tyrosinase

Tyrosinase solution (concentration 62.5 µg/ml) was exposed to longwave UV light (365 nm) for different time intervals and their respective fluorescence spectra was determined in the wavelength range 300 - 400 nm. The excitation wavelength was 280 nm. All the fluorescence spectra are shown in Figure 15. Different doses of UV irradiation bring a significant increase in fluorescence intensity of the enzyme molecule without any characteristic shift in the fluorescence maxima. The maximum increase in fluorescence of 34% was observed at a irradiation dose of 45.07×10^{19} Photons/cm^2.

2. Fluorescence Spectra of Psoralen

Upon irradiation of aqueous solution of psoralen by 365 nm light at different doses, the fluorescence spectra was determined (wavelength range 360 - 540 nm, excitation at 330 nm). The results are depicted in Figure 16. The characteristic maximum of psoralen at 450 nm was converted into two distinct maxima at 440 and 400 nm as a result of UV irradiation. The extent of relative fluorescence at 440 nm was increased with a short dose of 2.8×10^{19} Photons/cm^2. Further increase in irradiation dose have very little effect on the fluorescence. It should be noticed that entirely new fluorescence maximum near 400 nm emerged due to UV irradiation, the relative fluorescence at which increased with increasing dose without apparently having any effect on the native fluorescence of psoralen at 440 nm.

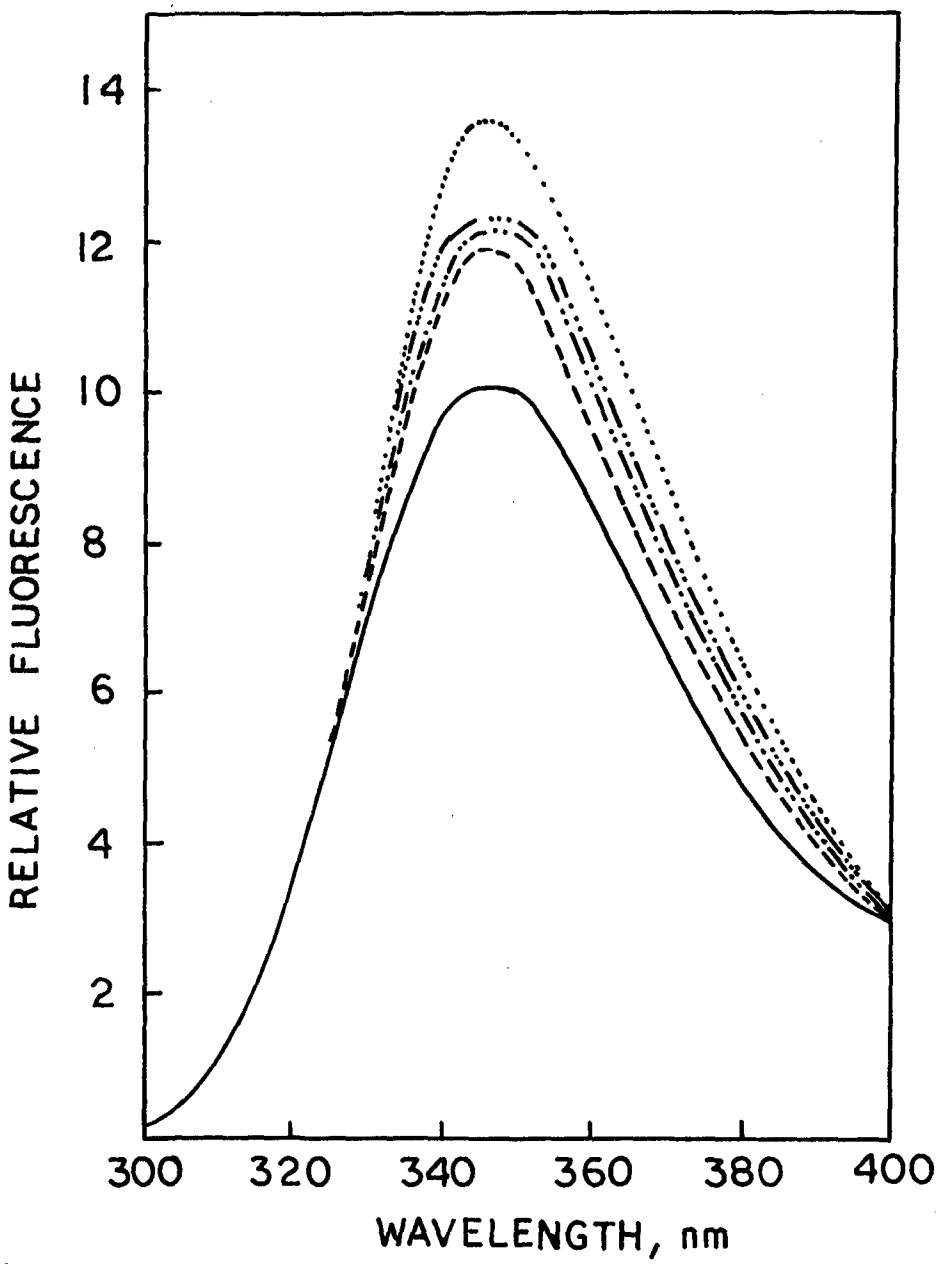


Figure 15. Fluorescence spectra of tyrosinase non-irradiated and irradiated by longwave UV light. Irradiation dose: (———) zero; (- - - - -) 5.6×10^{19} Photons/cm^2; (— · — · —) 16.9×10^{19} Photons/cm^2; (— · · — · · —) 22.5×10^{19} Photons/cm^2; (· · · · · · ·) 45.07×10^{19} Photons/cm^2. The concentration of tyrosinase was 62.5 µg/ml; excitation wavelength 280 nm.

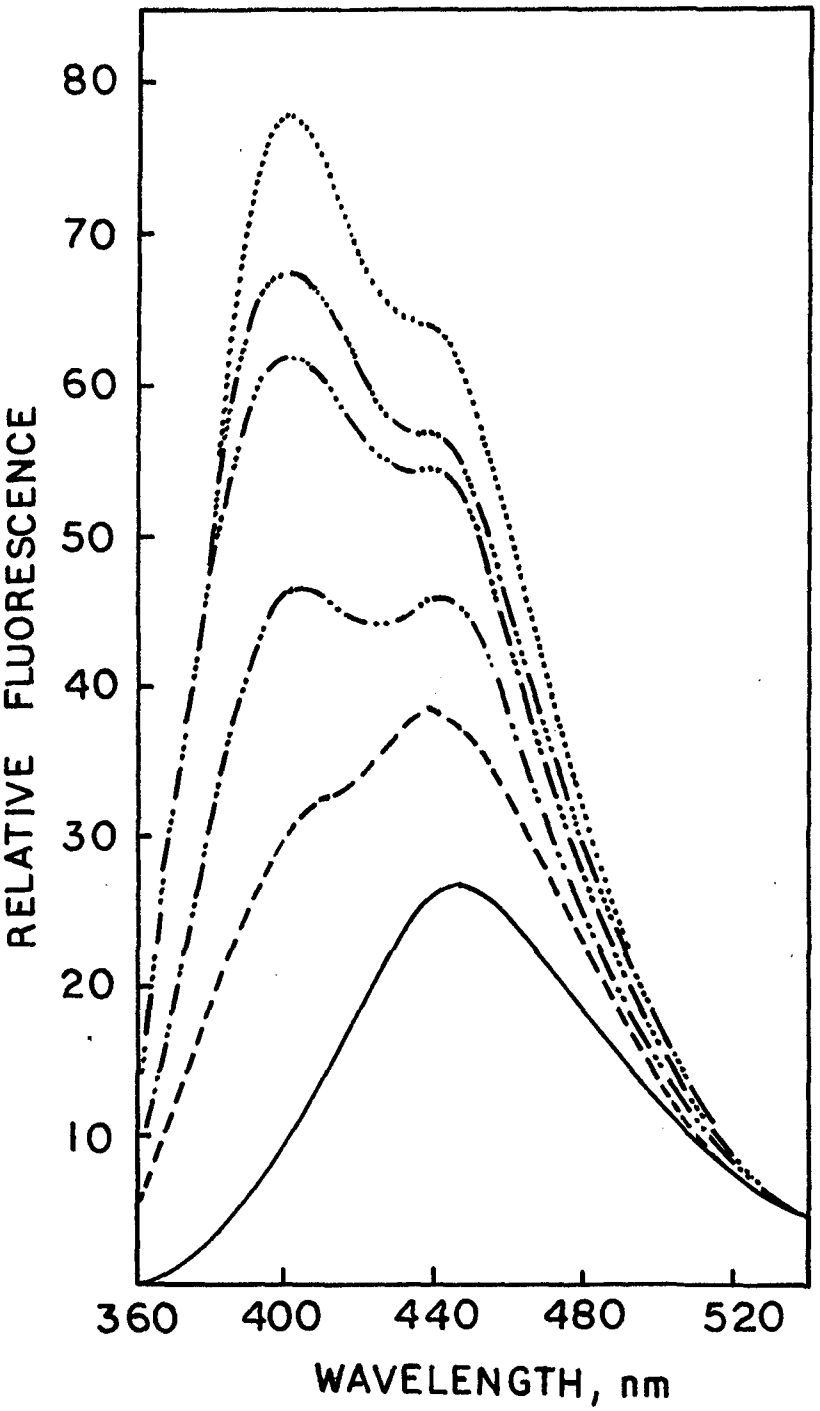


Figure 16. Fluorescence spectra of psoralen non-irradiated and irradiated by longwave UV light. Irradiation dose: (———) zero; (- - - - -) 2.81 x 10^{19} Photons/cm^2; (— · — · —) 8.44 x 10^{19} Photons/cm^2; (— · · — · · —) 14.07 x 10^{19} Photons/cm^2; (— · · · — · · · —) 19.7 x 10^{19} Photons/cm^2; (·····) 33.8 x 10^{19} Photons/cm^2. The concentration of psoralen was 25 µg/ml in 0.1 M phosphate buffer pH 6.5. The wavelength of excitation was 330 nm.

3. Interaction Between Psoralen and Tyrosinase

(i). Fluorescence Spectra

Tyrosinase (62.5 µg/ml) was irradiated for different doses, in presence of psoralen (25 µg/ml) and the relative fluorescence was monitered at 350 nm, excitation wavelength being 280 nm (Figure 17). The quantum yield of fluorescence of enzyme was increased when the enzyme was irradiated for varying doses and the maximum increase in fluorescence was about 35% at 45.07×10^{19} Photons/cm^2. In the presence of psoralen the fluorescence intensity of enzyme was markedly quenched, however, in this case no significant increase in fluorescence intensity of enzyme as a function of irradiation, was observed.

(ii). Activity of Tyrosinase

The biological activity of tyrosinase was studied in presence and absence of UV light, with and without psoralen using DL-Dopa as substrate (Figure 18). The catecholase activity of the enzyme was decreased by 16 per cent as a result of irradiation (33.8×10^{19} Photons/cm^2). The rate of loss of biological activity was calculated to be 0.05 per cent per minute. Under identical conditions, the enzyme activity was determined on irradiation in presence of psoralen. A sharp decrease in biological activity was observed initially (upto an irradiation dose of 11.2×10^{19} Photons/cm^2). Further increase in irradiation dose upto 45.07×10^{19} Photons/cm^2 results in a slow decrease in activity. The rate of loss of catecholase activity in this case was calculated to be 0.15 per cent per minute, which is three times faster

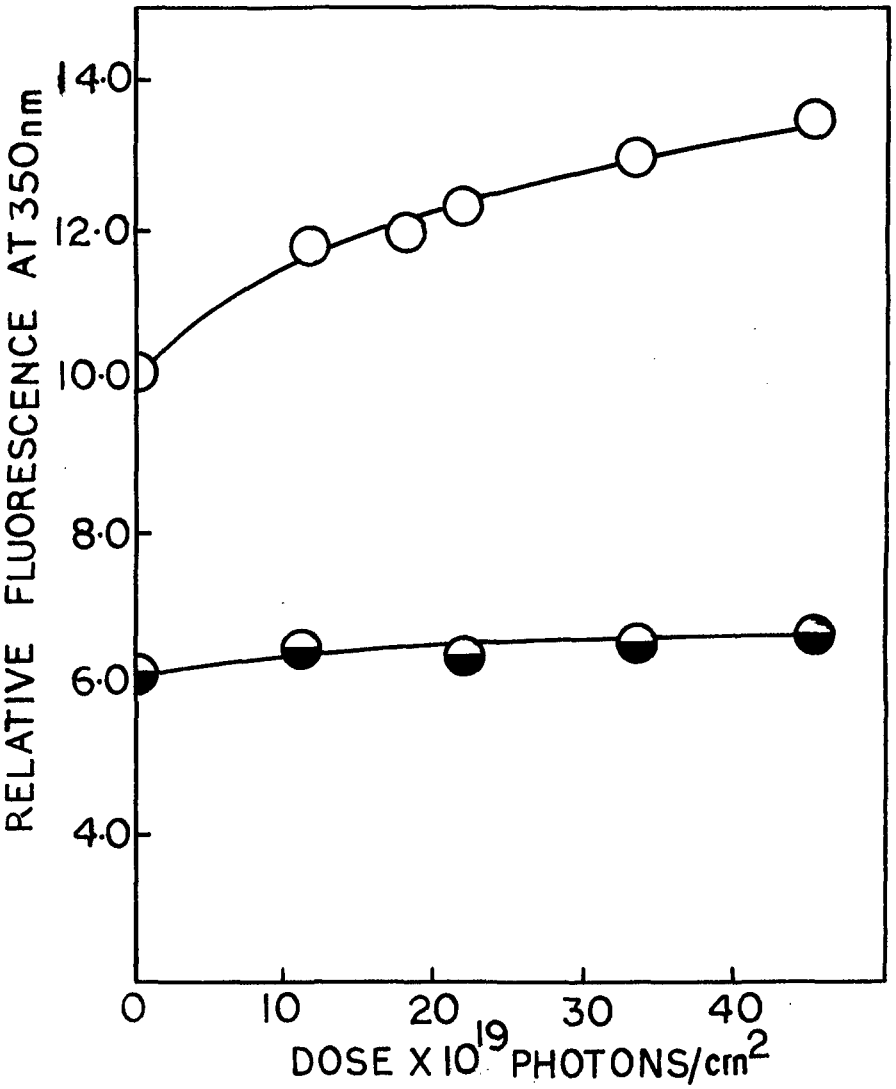


Figure 17. Effect of UV irradiation on the relative fluorescence of tyrosinase in presence (—●—) and absence (—○—) of psoralen. The concentrations of tyrosinase and psoralen were 62.5 µg/ml and 25 µg/ml respectively. Solutions were prepared in 0.1 M sodium phosphate buffer pH 6.5. Wavelength of excitation was 280 nm.

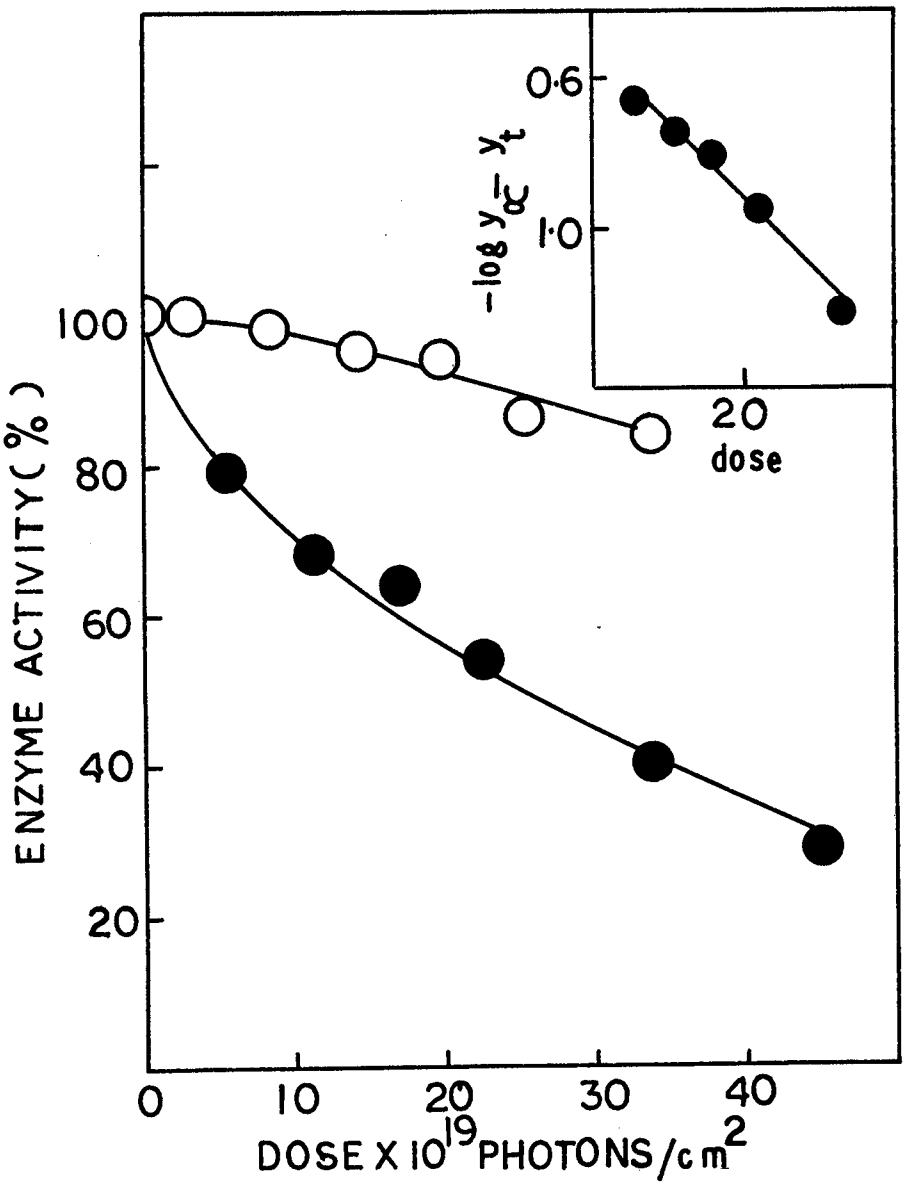


Figure 18. Effect of UV irradiation on tyrosinase activity in presence (—●—) and (—○—) absence of psoralen. Activity was measured using 2 mM dopa as substrate at 30°. Other experimental conditions were as in Figure 17. The inset shows the first order kinetic plot where y_∞ is the per cent activity at maximum irradiation dose and y_t is the per cent activity at any irradiation dose used in this experiment.

than the enzyme irradiated alone. Kinetic analysis of data showed that the loss in biological activity as a function of irradiation dose followed first order kinetics, as shown in the inset of Figure 18.

4. Effect of Psoralen Concentration on the Tyrosinase Activity in Presence of UV Light

From the earlier experiment it is clear that the enzyme activity was decreased when irradiated in presence of psoralen. Since tyrosinase show two different activities (cresolase and catecholase) the effect of irradiation and varying concentration of psoralen (4.1 to 25 µg/ml) was studied on both these activities (Figure 19 A and B). A significant decrease in cresolase activity was observed with increasing psoralen concentration at an irradiation dose of 7.5×10^{19} Photons/cm^2. A decrease in catecholase activity (Figure 19 B) was also observed with increasing psoralen concentration, however, under identical conditions the extent of decrease was appreciably less. Only 45 per cent loss in catecholase activity was noticed under maximum limit of irradiation and psoralen concentration. The loss in cresolase activity was 1.5 times than that of catecholase activity under these conditions.

5. Molar Ratio of Psoralen with Tyrosinase

The molar ratio of tyrosinase irradiated by ultraviolet light (7.5×10^{19} Photons/cm^2) with increasing concentration of psoralen was determined by fluorescence titration (Figure 20). The fluorescence intensity decreased abruptly during the initial increase in psoralen

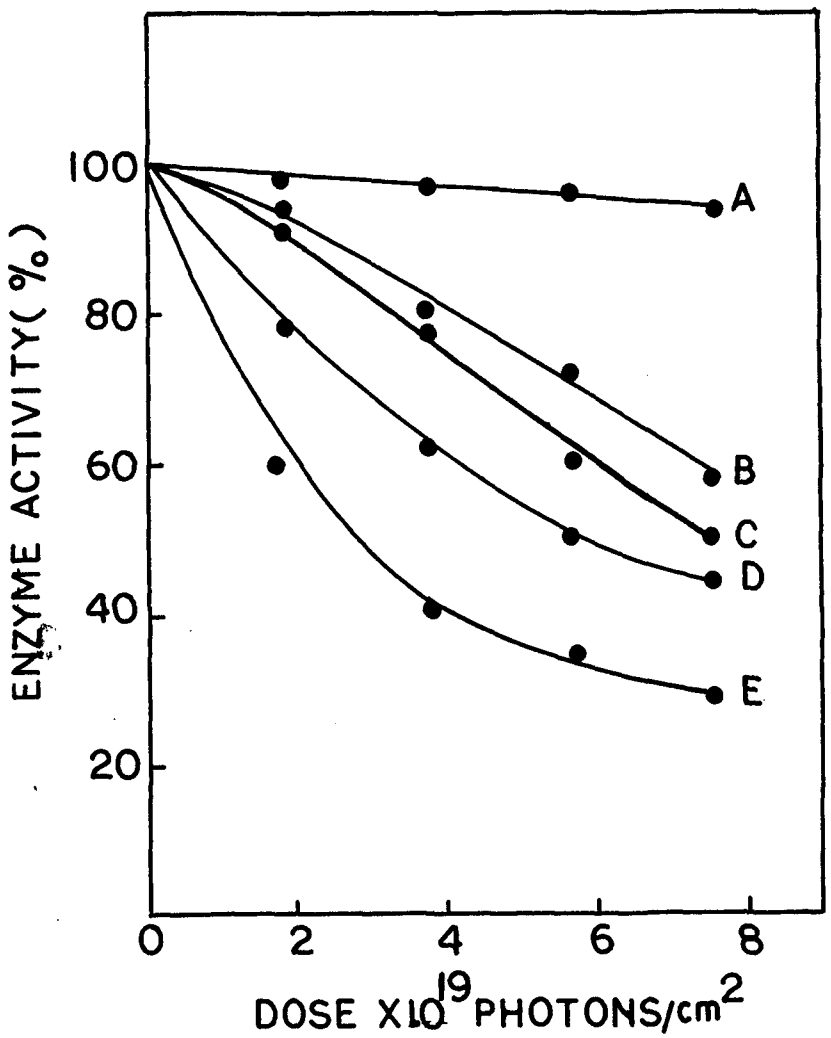


Figure 19. (A). Effect of psoralen on tyrosinase activity in presence of UV light. The concentration of psoralen was varied as follows: (A) zero; (B) 4.1 µg/ml; (C) 8.3 µg/ml; (D) 12.5 µg/ml; (E) 25.0 µg/ml. Activity was measured using 2 mM tyrosine as substrate. The solutions were prepared in 0.1 M sodium phosphate buffer pH 6.5.

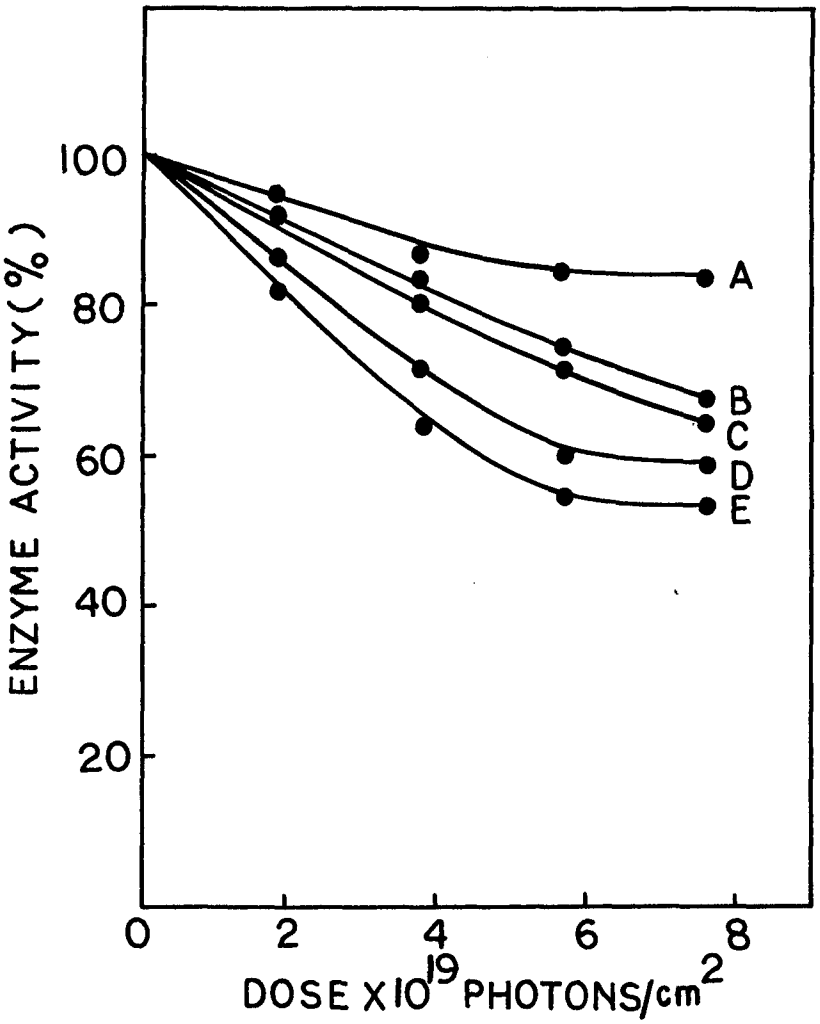


Figure 19. (B). Effect of psoralen on tyrosinase activity in presence of UV light. The concentration of psoralen was varied as follows: (A) zero; (B) 4.1 µg/ml; (C) 8.3 µg/ml; (D) 12.5 µg/ml; (E) 25.0 µg/ml. Activity was measured using 2 mM dopa as substrate. Other experimental conditions were similar to that of Figure 19 (A).

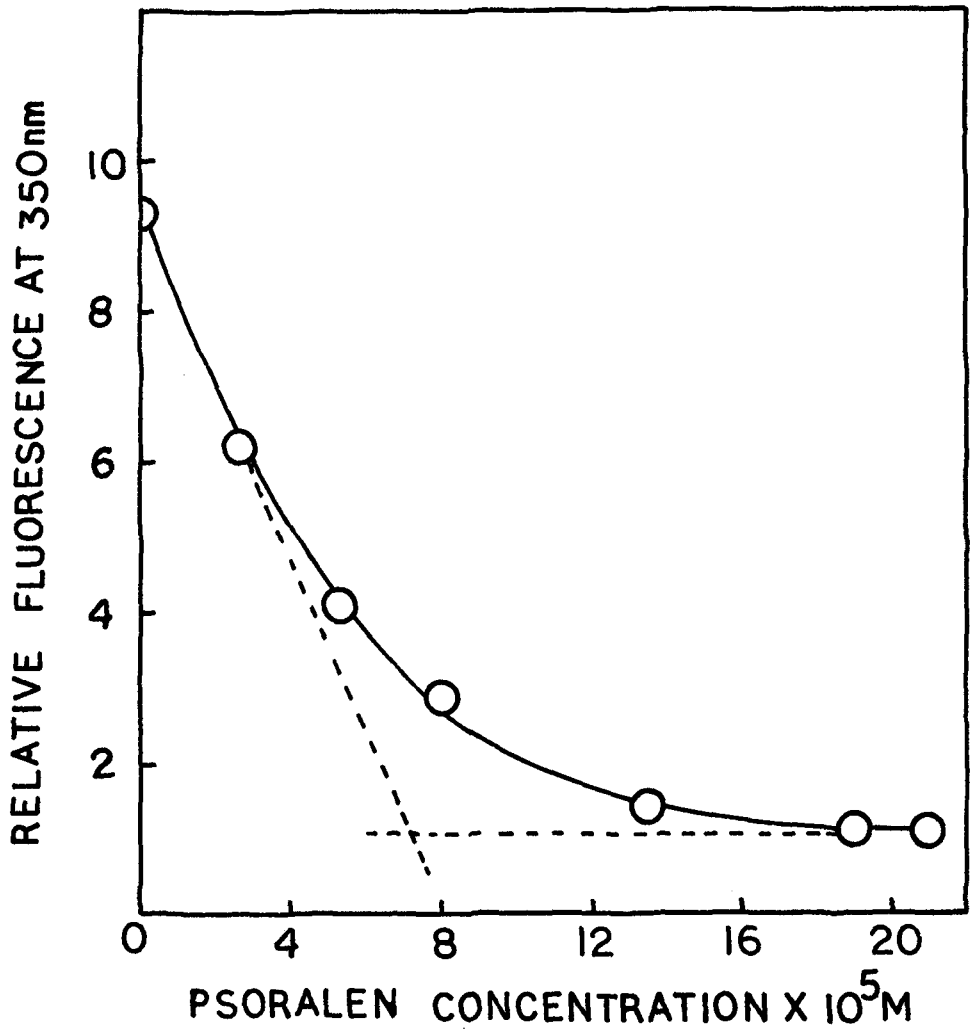


Figure 20. Fluorometric titration of irradiated tyrosinase. Enzyme was irradiated by longwave UV light (dose; 7.5×10^{19} Photons/cm^2) in 0.1 M sodium phosphate buffer pH 6.5, μ 0.18. To a fixed concentration (200 μg/ml) of irradiated enzyme solution, increasing concentrations of psoralen was added and the fluorescence was recorded. The enzyme concentration at the point of intersection was 7.8×10^{-7} M. Excitation wavelength was 280 nm.

concentration thereafter become constant. Two lines of the curve were extrapolated and the point of intersection was computed to 7.2×10^{-5} M psoralen. Thus the molar ratio between psoralen and tyrosinase under the influence of longwave ultraviolet light was found to be 92.

III. KINETIC PARAMETERS IN PRESENCE AND ABSENCE OF ULTRAVIOLET LIGHT

1. Michaelis Menten Constant

In order to characterize the affinity of the enzyme for its substrates (tyrosine and dopa) in presence and absence of UV light with and without psoralen, effect of substrate concentration on the cresolase and catecholase activities was studied. Results are given in Table IV and Lineweaver Burk plots in Figure 21 to 24.

Psoralen per se had no effect on association constant of enzyme with tyrosine as evident from the Km value being 2.2×10^{-4} M in the presence and absence of drug. However, Km for dopa under these experimental conditions were 4.5×10^{-4} M and 0.3×10^{-4} M in absence and presence of drug respectively. On irradiation of enzyme by ultraviolet light of 365 nm, the value of Km was significantly decreased for both the substrates, the decrease being 1.5 and 2.6 fold for tyrosine and dopa respectively. Surprisingly the Km for dopa, when tyrosinase was irradiated in presence of psoralen, remained unchanged while 4.4 fold decrease in Km for tyrosine was noticed.

2. Inhibitor Constant

The effect of seven non-competetive inhibitors on the biological

TABLE IV

EFFECT OF UV IRRADIATION AND PSORALEN ON MICHAELIS-MENTEN CONSTANT FOR CRESOLASE AND CATECHOLASE ACTIVITY OF TYROSINASE

Enzyme concentration 1.56×10^{-7} moles/l pH 6.5

Irradiation dose 7.5×10^{19} Photons/cm^2 Ionic strength 0.05

Psoralen concentration 4.2 µg/ml

	Km (10^{4}M)			
	Cresolase activity*		Catecholase activity**	
	- Psoralen	+ Psoralen	- Psoralen	+ Psoralen
Non-irradiated	2.2	2.2	8.3	4.5
Irradiated	1.4	0.5	3.3	5.0

* Tyrosine as substrate,

** DL-Dopa as substrate.

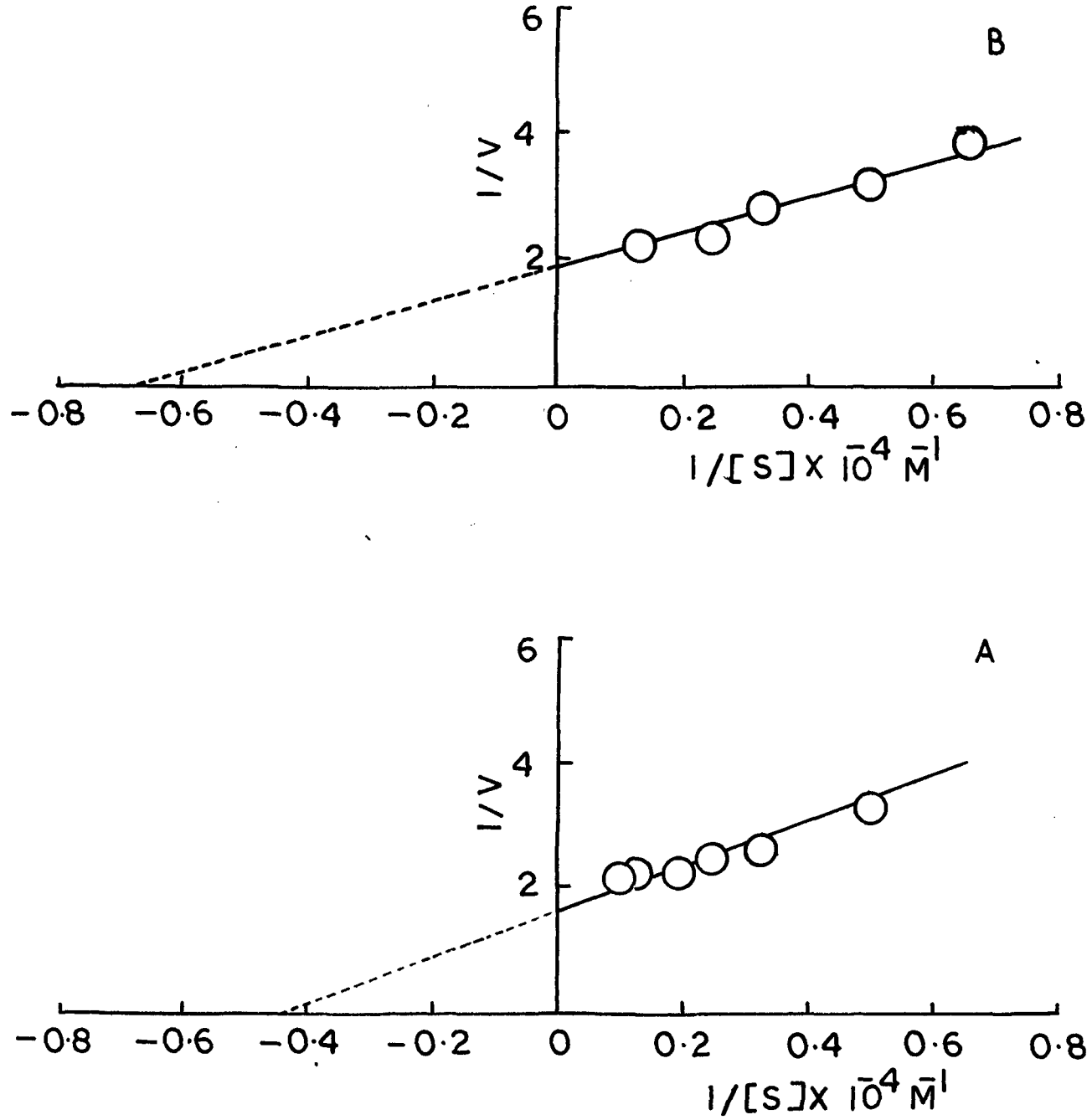


Figure 21. Km for tyrosinase; (A) non-irradiated, (B) irradiated. The enzyme concentration was 1.56×10^{-7} M and substrate (tyrosine) concentration varied from 0.1 to 1.0 mM. The solutions were prepared in 0.1 M sodium phosphate buffer pH 6.5. Irradiation dose was 7.5×10^{19} Photons/cm^2. Velocities were expressed as absorbance at 480 nm.

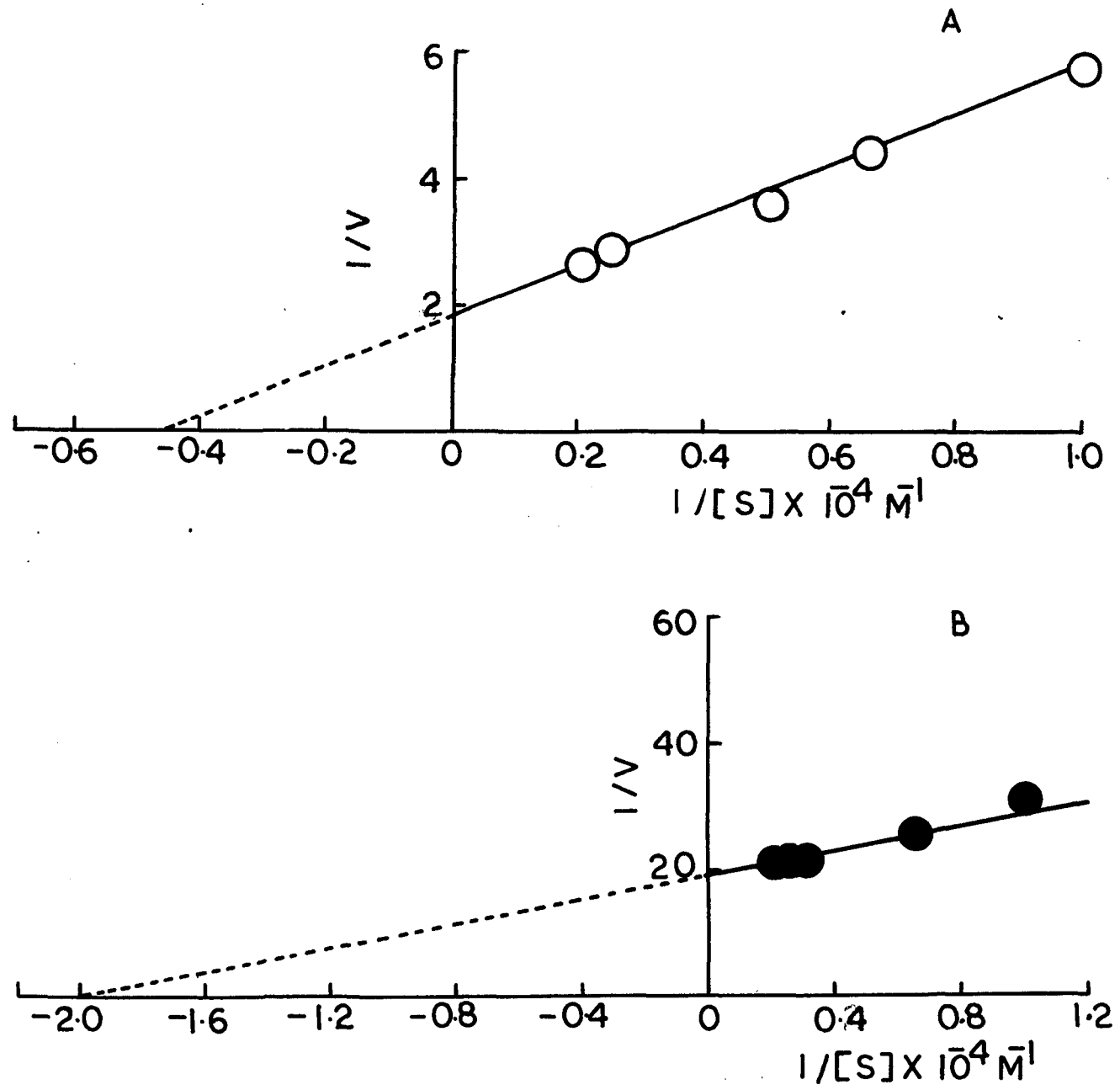


Figure 22. Km for tyrosinase in presence of psoralen; (A) non-irradiated, (B) irradiated. The concentration of psoralen was 4.2 µg/ml. Other experimental details as in Figure 21.

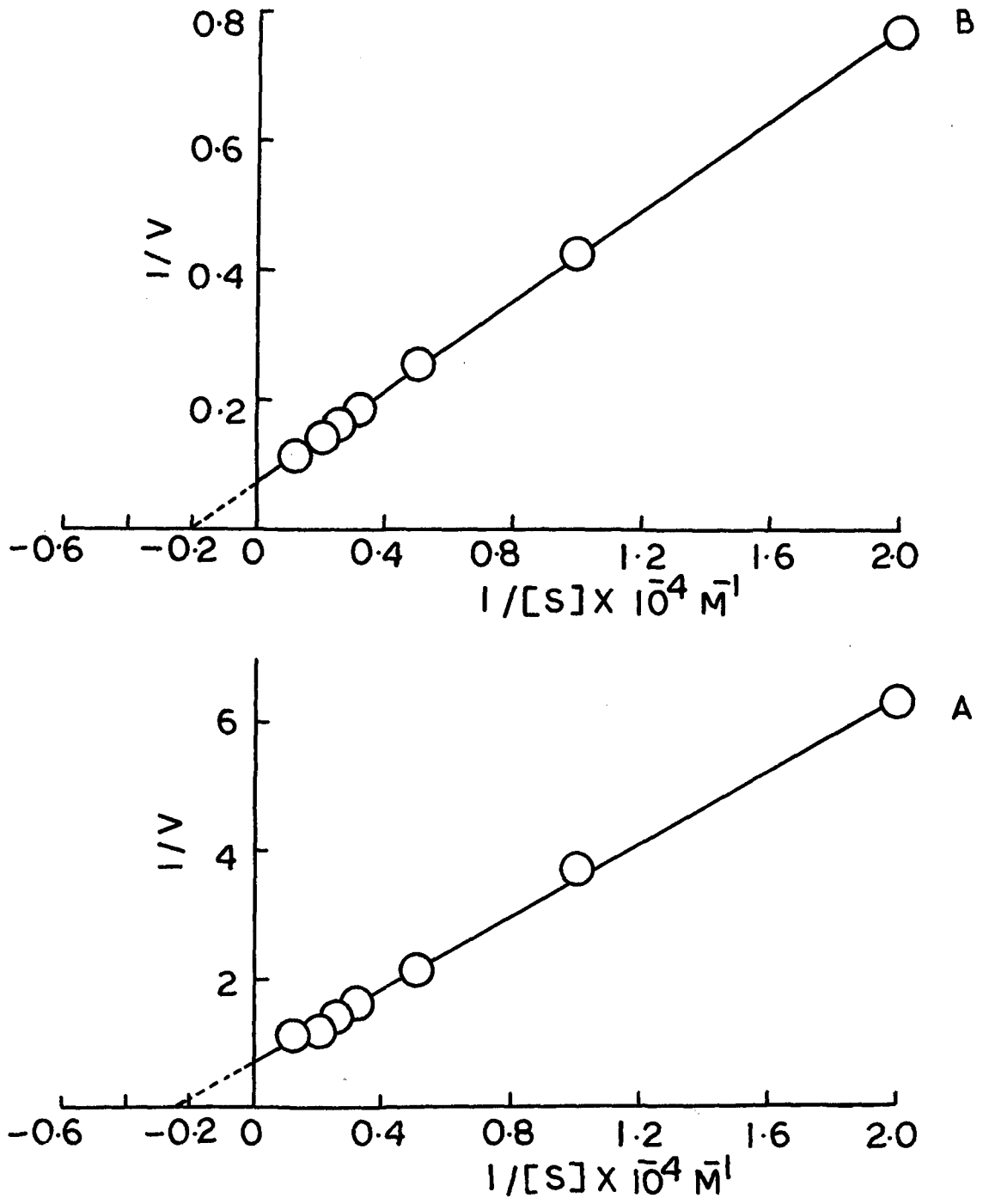


Figure 23. Km for tyrosinase; (A) non-irradiated, (B) irradiated. The concentration of enzyme was 1.56×10^{-7} M and that of substrate (dopa) varied from 0.05 - 0.7 mM. The solutions were prepared in 0.1 M phosphate buffer pH 6.5. Irradiation dose was 2.5×10^{19} Photons/cm^2. Velocities were expressed as absorbance at 480 nm.

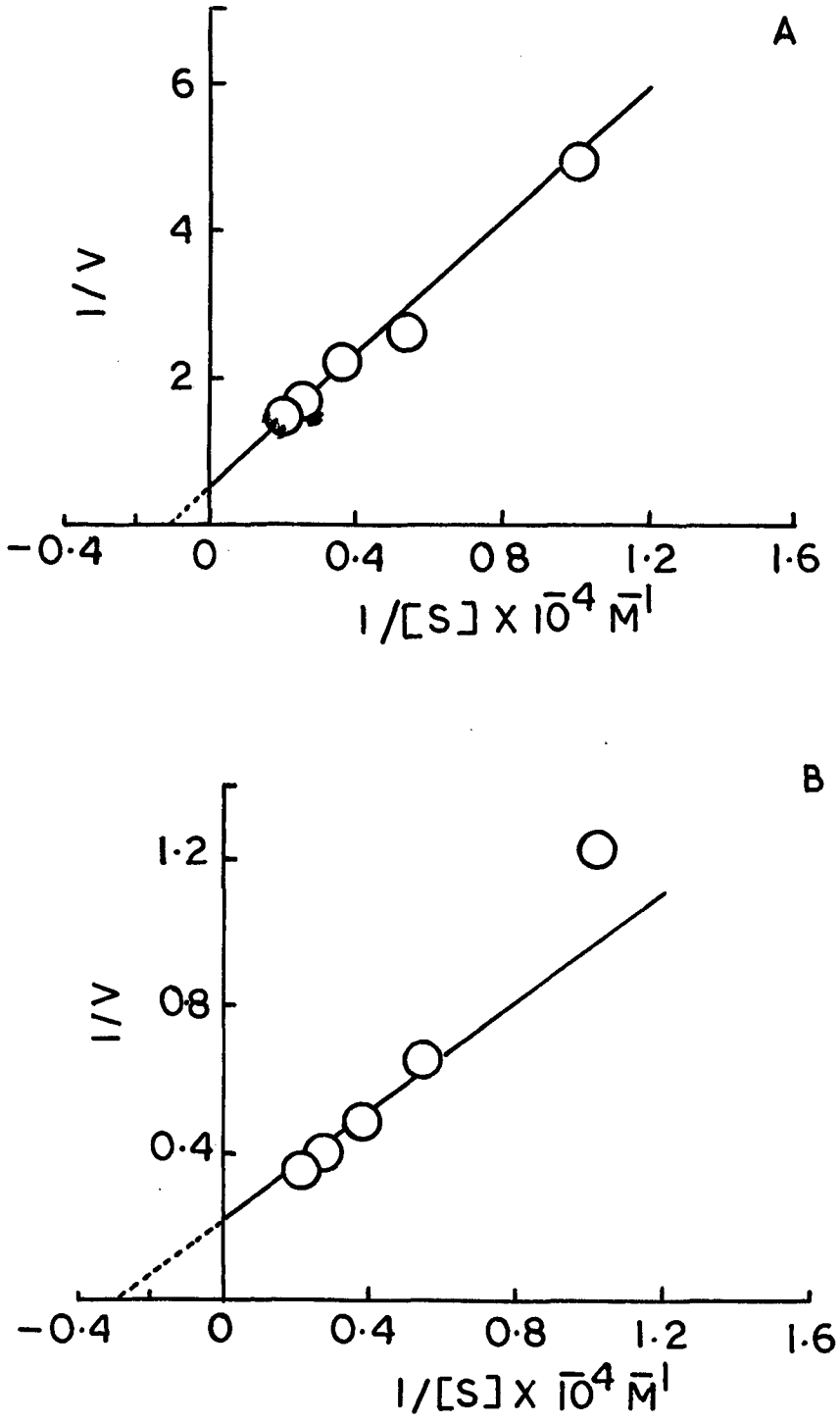


Figure 24. Km for tyrosinase in presence of psoralen; (A) non-irradiated, (B) irradiated. The concentration of psoralen was 4.2 µg/ml. Other experimental conditions as in Figure 23.

activity of native and irradiated tyrosinase in presence and absence of psoralen was studied and the results are given in Table V. It is indicated from the data that per cent inhibition of tyrosinase activity for different inhibitors was generally decreased when the enzyme was irradiated. Similarly a decrease in per cent inhibition of tyrosinase activity was also observed when it was irradiated in the presence of psoralen, however, in this case extent of decrease was comparatively less.

The value of Ki was determined for thiouracil and glutathione in presence of psoralen and subsequent irradiation (Table VI). The experimental observations were plotted according to the method of Lineweaver and Burk (70) and the value of Ki was computed from the slope of the straight lines (Figure 25, 26). For thiouracil, in presence and absence of psoralen, the Ki was 3×10^{-4} M. However, Ki was increased to 11.8×10^{-4} M on irradiation by UV light. Interestingly in presence of psoralen and subsequent irradiation, the Ki was in significantly decreased.

In case of glutathione, the value of Ki was increased from 6.5×10^{-4} to 16.1×10^{-4} M on irradiation by UV light. It is worth to mention the increase in Ki for thiouracil, after irradiation, was relatively higher than that for glutathione.

TABLE V

EFFECT OF INHIBITORS ON TYROSINASE ACTIVITY

Tyrosinase concentration
2.6×10^{-7} moles/l

Psoralen concentration
6.25 µg/ml.

Inhibitor concentration 1 mM

pH 6.5

Ionic strength 0.18

Irradiation dose
7.5×10^{19} Photons/cm^2

	Inhibition (%)*			
	- Psoralen		+ Psoralen	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
Diethyldithio-carbamate	91.4	41.5	48.4	44.0
Glutathione	96.8	55.6	95.2	79.0
Thiouracil	74.8	50.0	79.3	68.8
Cysteine	96.4	56.6	96.6	82.0
Ascorbic acid	96.4	55.6	95.0	78.8
Sodium azide	46.2	43.4	57.5	58.0
2-mercaptoethanol	96.8	55.6	96.6	78.8

* Enzyme was incubated with different inhibitors for one hour and then the activity was measured using Tyrosine (2 mM) as substrate.

TABLE VI

INHIBITOR CONSTANT FOR TYROSINASE

Enzyme concentration 1.56×10^{-7} moles/l — Substrate Tyrosine

Inhibitor concentration 2.5×10^{-4} M — pH 6.5

Psoralen concentration 6.2 µg/ml — Temperature 30°

	Ki (10^4 M)		
	Thiouracil		Glutathione
	- Psoralen	+ Psoralen	- Psoralen
Non-irradiated	3.3	2.9	6.5
Irradiated*	11.8	1.72	16.1

* Irradiation dose 7.5×10^{19} Photons/cm^2.

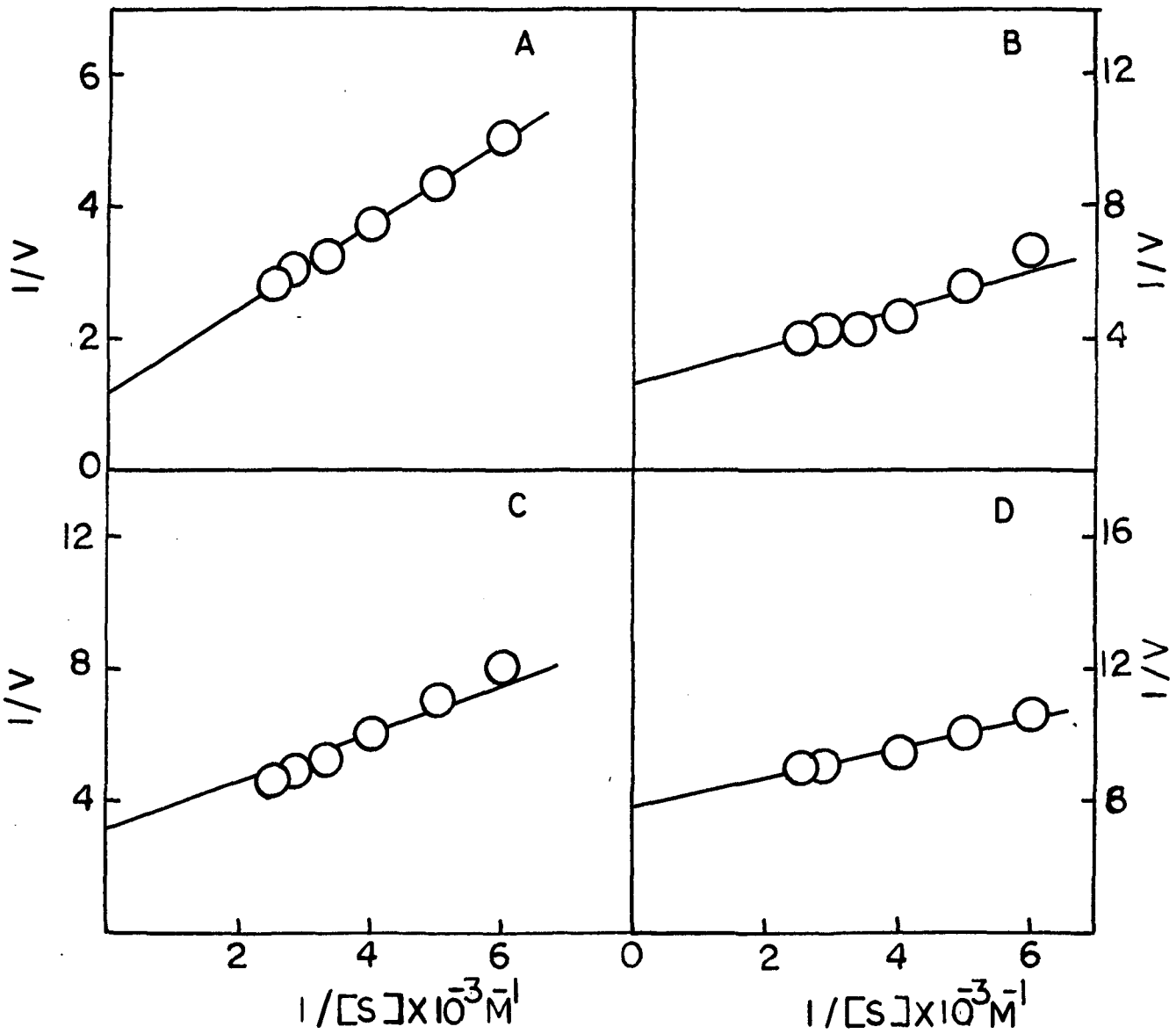


Figure 25. Inhibition of tyrosinase by thiouracil. The concentrations of enzyme, thiouracil and psoralen were 1.56×10^{-7} M, 2.5×10^{-4} M and 6.2 µg/ml, respectively. The concentration of substrate (tyrosine) varied from 0.05 mM to 0.40 mM. The solutions were prepared in 0.1 M phosphate buffer pH 6.5. (A) non-irradiated tyrosinase; (B) irradiated tyrosinase; (C) non-irradiated tyrosinase in presence of psoralen; (D) tyrosinase irradiated in presence of psoralen.

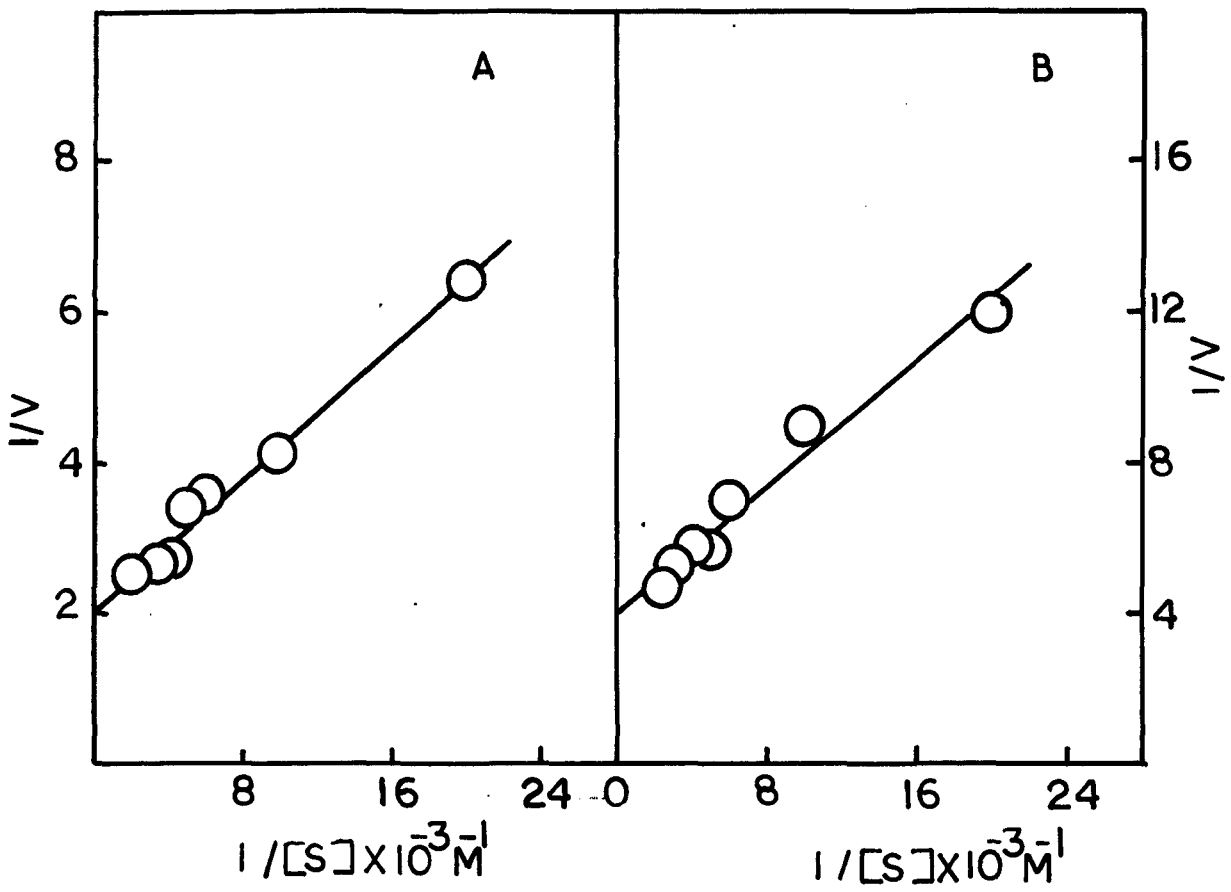


Figure 26. Inhibition of tyrosinase by glutathione. The concentration of glutathione was 2.5×10^{-4} M. Other experimental conditions were as in Figure 25. (A) non-irradiated tyrosinase (B) irradiated tyrosinase.

IV. EFFECT OF LONGWAVE ULTRAVIOLET LIGHT ON INHIBITORS OF TYROSINASE

1. Ultraviolet Absorption Spectra

A. Thiouracil

Thiouracil, one of the inhibitors of tyrosinase, was irradiated for different doses and UV absorption spectra were recorded (Figure 27). Spectra were characterized with a maximum near 277 nm and a sharp trough near 242 nm. Increasing dose of irradiation reflected by a slight increase in absorbance near 277 nm without any shift in maximum. However, UV absorption spectra of thiouracil irradiated in presence of different doses (Figure 28) indicate a significant decrease in absorbance at 272 nm.

B. Diethyldithiocarbamate

The ultraviolet absorption spectrum of diethyldithiocarbamate has two characteristic maxima at 258 and 281 nm and two distinctive troughs at 236 and 270 nm (Figure 29). As a result of its irradiation for different doses a notable perturbation in spectrum was observed and the magnitude of absorption at its maxima (258 nm and 281 nm) was significantly decreased. Irradiation for a higher dose (22.53×10^{19} Photons/cm^2) results in the complete disappearance of the fine structure of spectrum thereby a plateu region in the wavelength 258 nm to 281 nm developed. A slight shift of 4 nm in trough at 236 nm was also observed. Irradiation of diethyldithiocarbamate in the presence of psoralen (16.90×10^{19} Photons/cm^2) was characterized by a phenomenal increase

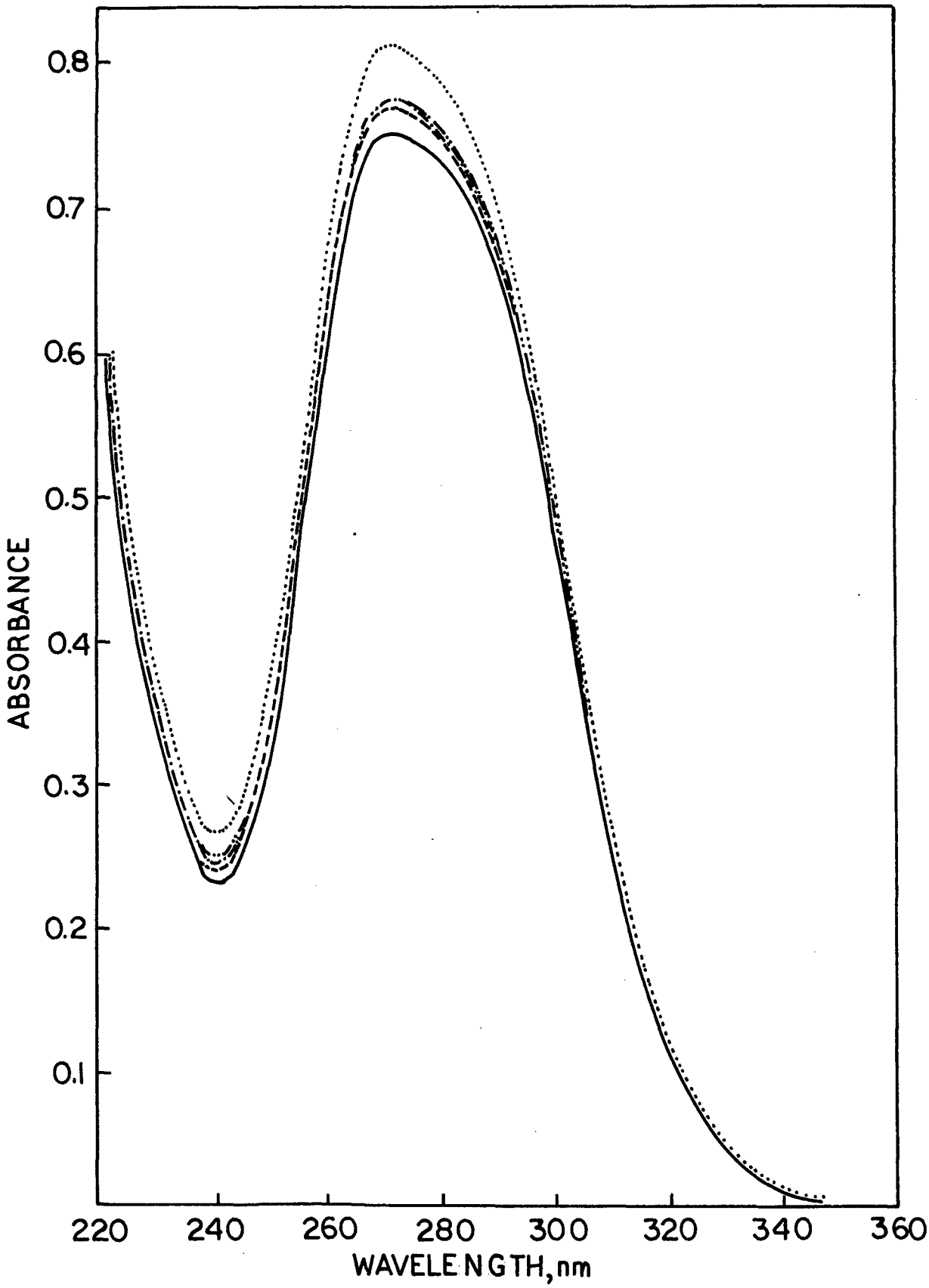


Figure 27. Effect of UV irradiation on thiouracil. The concentration of thiouracil was 0.05 mM, in 0.1 M sodium phosphate buffer pH 6.5. Irradiation dose: (———) zero; (- - - - -) 11.27×10^{19} Photons/cm^2; (—·—·—) 22.53×10^{19} Photons/cm^2; (——·—·—) 33.8×10^{19} Photons/cm^2 and (·····) 45.07×10^{19} Photons/cm^2.

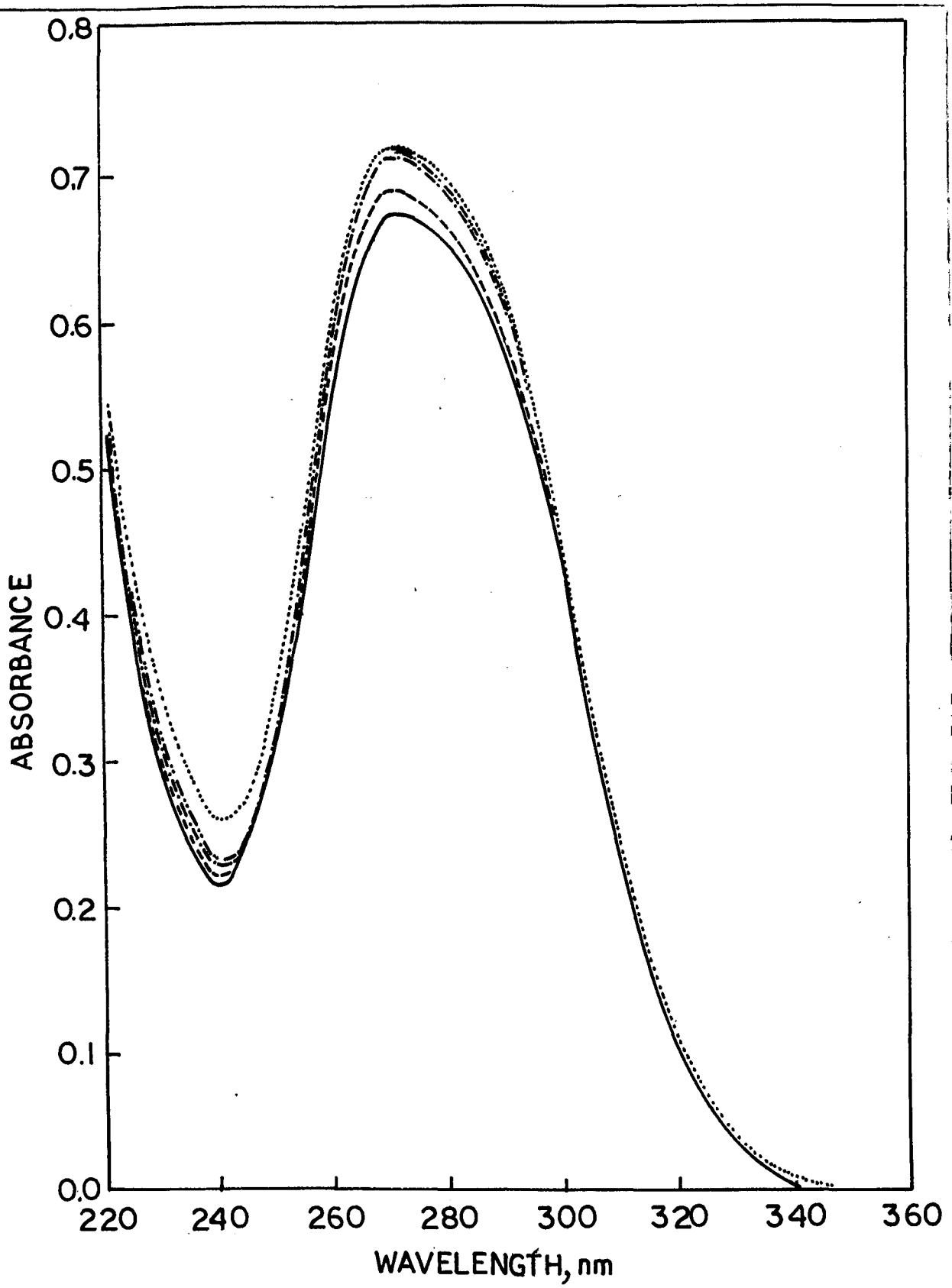


Figure 28. Effect of UV irradiation on thiouracil in presence of psoralen. Thiouracil (0.05 mM) with psoralen (10 µg/ml) in 0.1 M phosphate buffer pH 6.5, was irradiated for different doses: (.....) zero; (—·—·—) 11.27 x 10^{19} Photons/cm^2; (—·—·—) 22.53 x 10^{19} Photons/cm^2; (-----) 33.8 x 10^{19} Photons/cm^2 and (———) 45.07 x 10^{19} Photons/cm^2. The absorption by psoralen was overcome by taking corresponding control samples of psoralen as blanks.

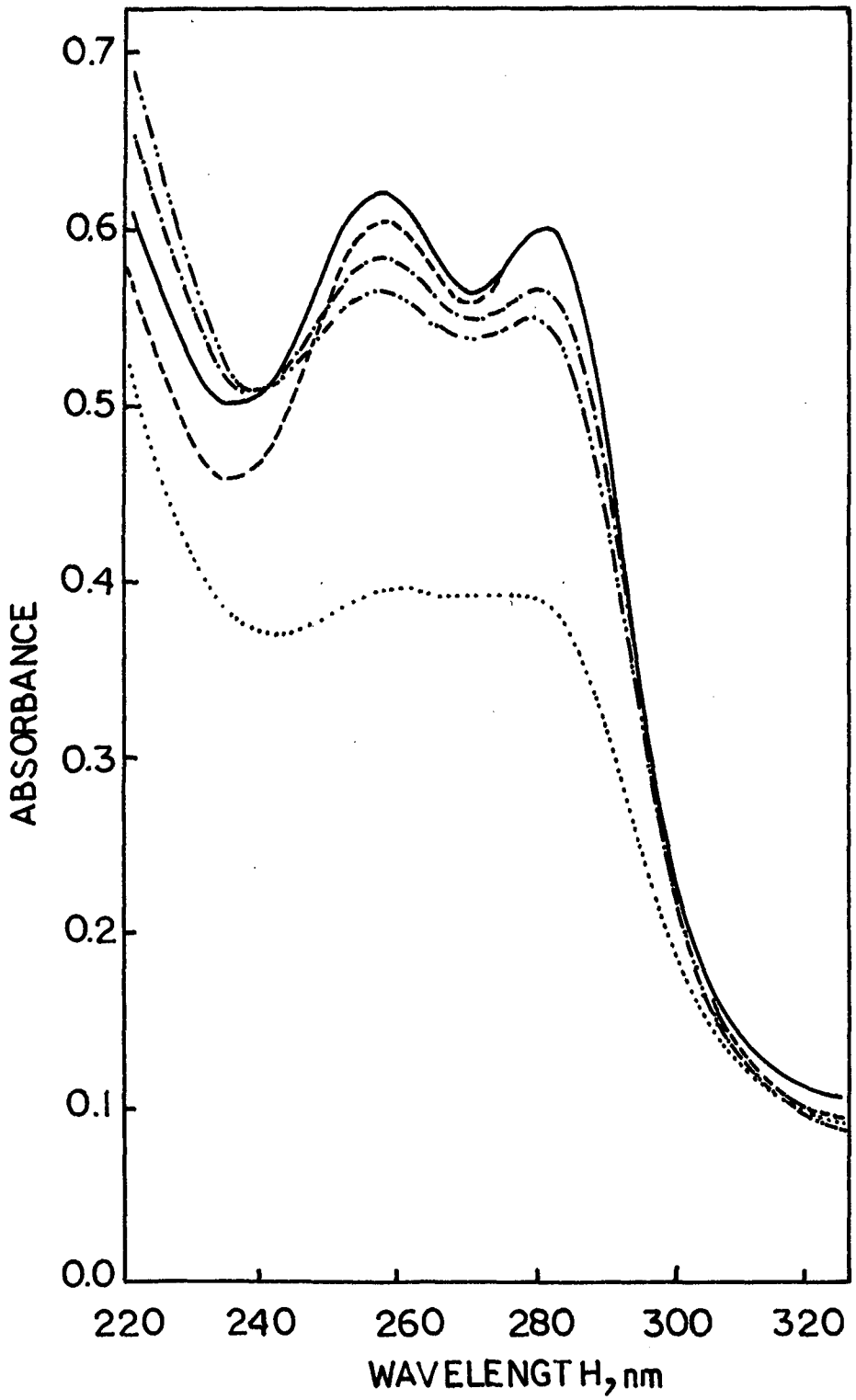


Figure 29. Effect UV irradiation on diethyldithiocarbamate. The concentration of diethyldithiocarbamate was 0.05 mM in 0.1 M phosphate buffer pH 6.5. Irradiation dose: (_____) zero; (- - - - - -) 5.6 x 10^{19} Photons/cm^2; (— — —) 11.27 x 10^{19} Photons/cm^2; (— · —) 11.27 x 10^{19} Photons/cm^2; (— · · —) 16.9 x 10^{19} Photons/cm^2; (· · · · · · · · ·) 22.53 x 10^{19} Photons/cm^2.

in absorption and a complete change in the characteristic spectra of the compound (Figure 30). The changes observed are the disappearance of one of its maximum at 258 nm, shifting of maxima at 281 nm to a broad absorption at 275 nm and a shift of 10 nm in trough at 236 nm to 246 nm. On increasing the irradiation dose (28.17×10^{19} Photons/cm^2), the extent of absorption was found to decrease which might be due to the decomposition of the complex formed between psoralen and diethyldithiocarbamate as a result of initial irradiation.

2. Biological Activity of Tyrosinase in Presence of Irradiated Inhibitors

The inhibiting capacity of variety of inhibitors of tyrosine-tyrosinase system irradiated with and without psoralen was studied (Table VII). The inhibitary capacity, of thiouracil, glutathione, cysteine and diethyldithiocarbamate on irradiation in presence and absence of psoralen, was not influenced, inspite of a significant change in the absorption spectra of thiouracil and diethyldithiocarbamate as a result of irradiation.

V. CONFORMATION OF ENZYME

1. Polyacrylamide Gel Electrophoresis

The results of polyacrylamide gel electrophoresis at pH 8.3 of native and irradiated tyrosinase in presence and absence of psoralen are shown in Figure 31, and their relative mobilities in Table VIII. A single, sharp disc of tyrosinase under different experimental conditions

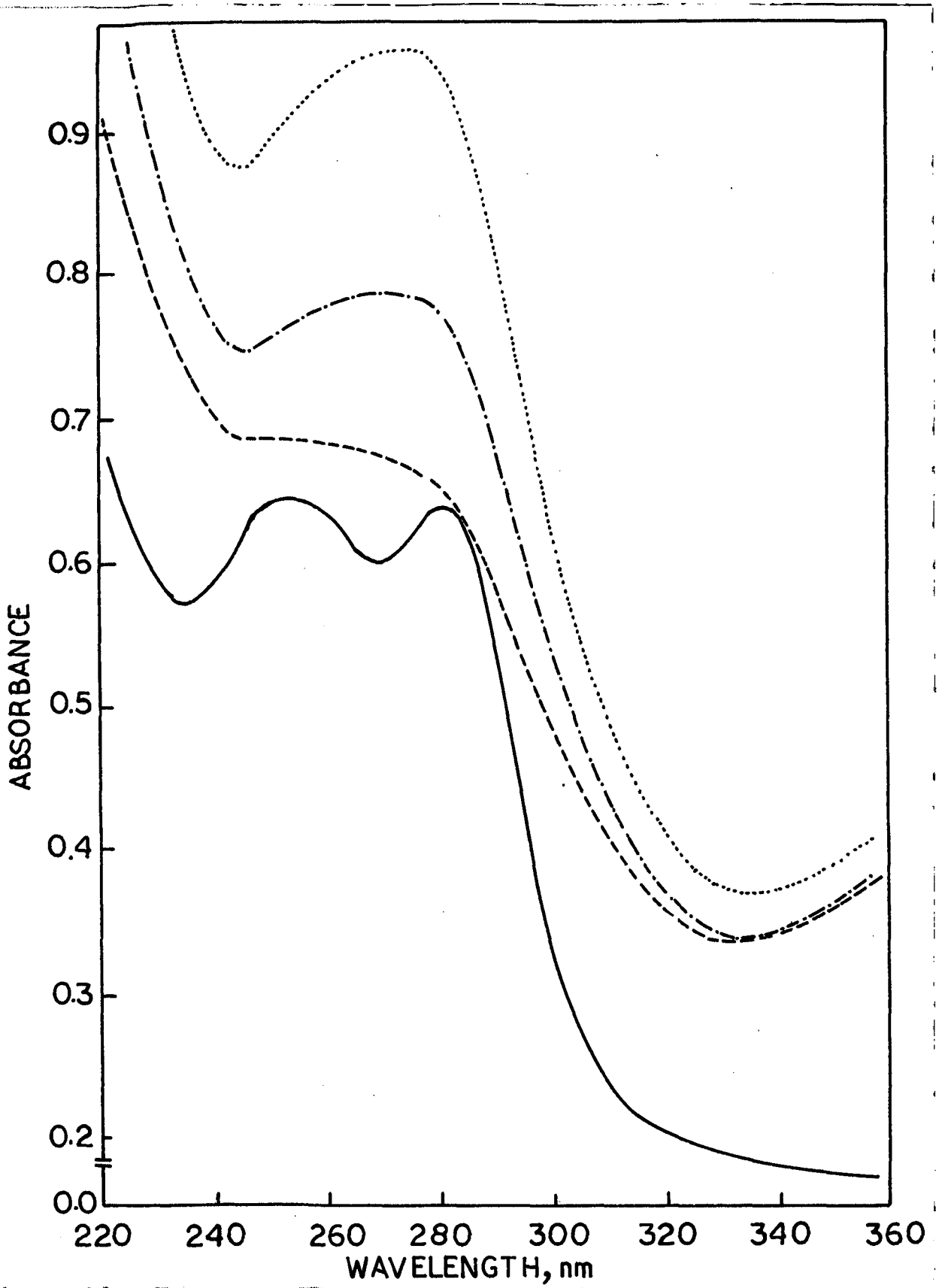


Figure 30. Effect of UV irradiation on diethyldithiocarbamate in presence of psoralen. The concentrations of diethyldithiocarbamate and psoralen were 0.05 mM and 10 µg/ml respectively in 0.1 M sodium phosphate buffer, pH 6.5. The absorption by psoralen was overcome by taking corresponding control samples of psoralen as blanks. Irradiation dose: (———) zero; (- - - - - -) 28.17×10^{19}; (— · — · —) 22.53×10^{19}; (·····) 16.90×10^{19} Photons/cm^2.

TABLE VII

EFFECT OF UV IRRADIATED INHIBITORS ON TYROSINASE ACTIVITY

Inhibitor concentration 1 mM — Tyrosinase concentration 2.6×10^{-7} moles/l

pH 6.5 — Substrate Tyrosine (2 mM)

Irradiation dose (10^{19} Photons/cm^2)	Inhibition (%)							
	Thiouracil		DDTC*		Glutathione		Cysteine	
	- P	+ P	- P	+P	- P	+ P	- P	+ P
0	75	78	90	50	96	96	96	97
11.26	76	77.5	91	51	97	97.5	97.8	97
22.53	74	77	92	50.5	96	95	98	96
33.80	75.8	78	90.5	52	96	95.5	97.5	96
45.07	77	78	90	52	96.5	95	96	96.5

* Diethyldithiocarbamate; + P, in presence of psoralen; - P, in absence of psoralen.

TABLE VIII

Rm VALUES OF TYROSINASE

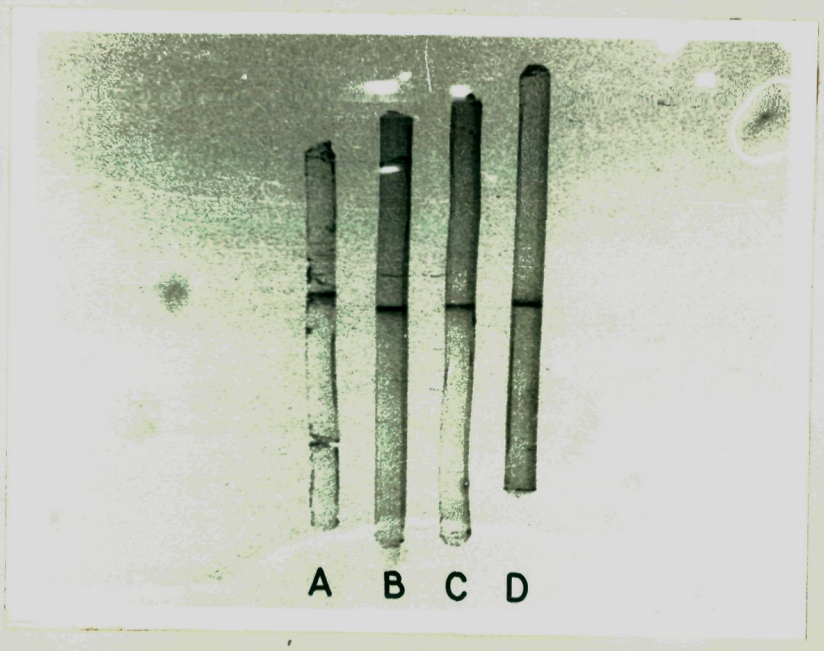
Tyrosinase applied 100 µg pH 8.3

Direction of flow of current from -ve to +ve

	Rm Values
Tyrosinase (Non-irradiated)	0.31
Tyrosinase (Irradiated)*	0.45
Tyrosinase + Psoralen (Non-irradiated)	0.46
Tyrosinase + Psoralen (Irradiated)	0.55

* Irradiation dose 7.5×10^{19} Photons/cm^2.

Figure 31. Polyacrylamide gel electrophoresis of tyrosinase (A) non-irradiated tyrosinase; (B) irradiated tyrosinase; (C) non-irradiated tyrosinase in presence of psoralen; (D) tyrosinase irradiated in presence of psoralen. Irradiation dose was 7.5×10^{19} Photons/cm^2. Electrophoresis was performed in 0.05 M borate buffer pH 8.3.


A
B
C
D

was observed. However, the relative mobility of the enzyme was increased after irradiation in presence and absence of psoralen.

2. Susceptibility of Tyrosinase to Tryptic Digestion

Irradiation of tyrosinase solution by 365 nm light increases the susceptibility towards tryptic digestion, reflected in terms of increase in absorption at 570 nm (Figure 32). A 12 per cent higher value in case of irradiated protein was noticed when compared with normal control. The tryptic susceptibility of tyrosinase in the presence of psoralen was significantly decreased.

VI. IMMUNOLOGICAL CHARACTERIZATION OF ENZYME

The cross reactivity of antibody raised against native tyrosinase was checked with native enzyme and with irradiated tyrosinase in presence and absence of psoralen (Figure 33). It could be concluded from the immunodiffusion pattern that irradiated tyrosinase in presence and absence of psoralen did not cross react with the antibody raised against native enzyme.

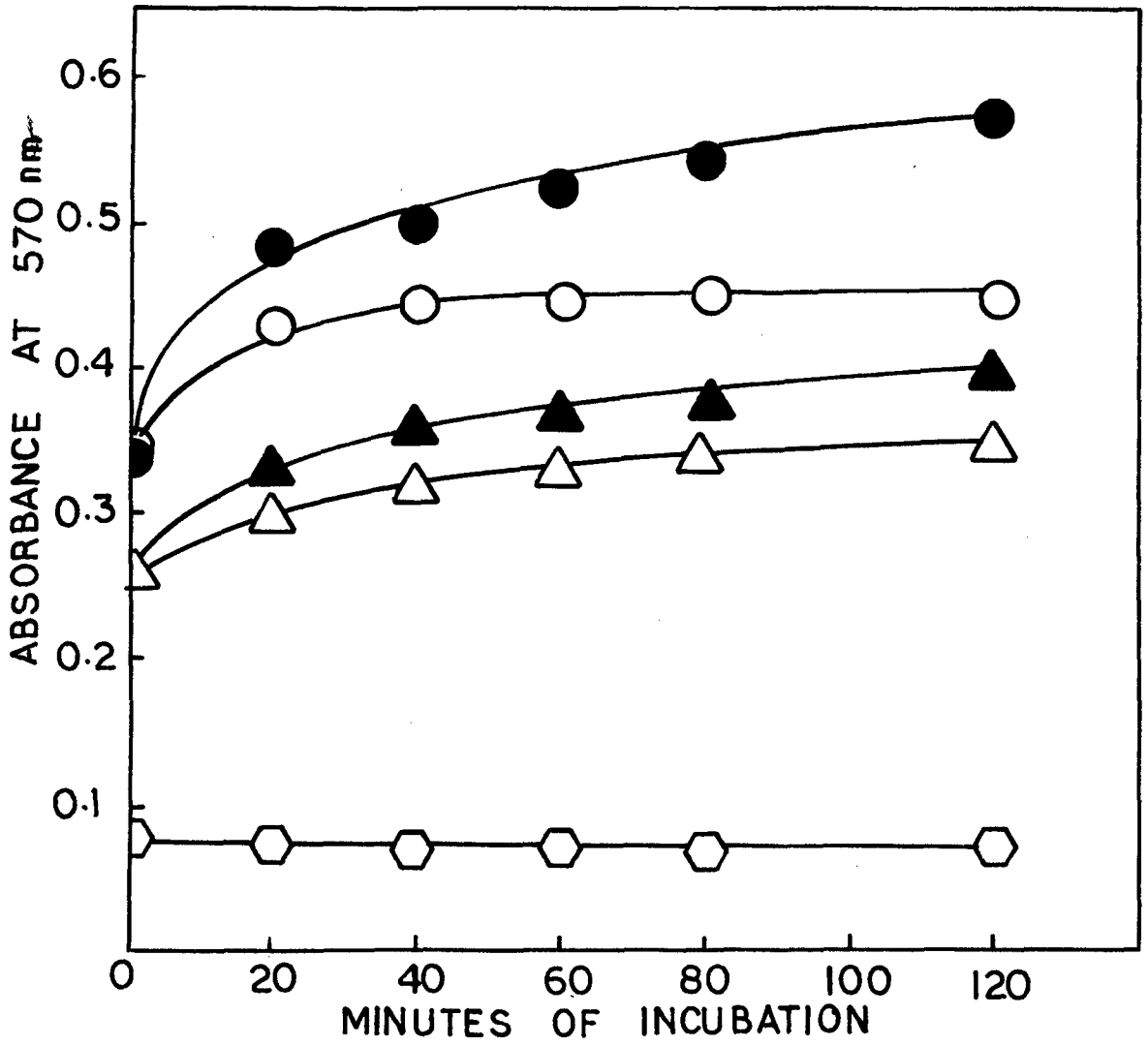
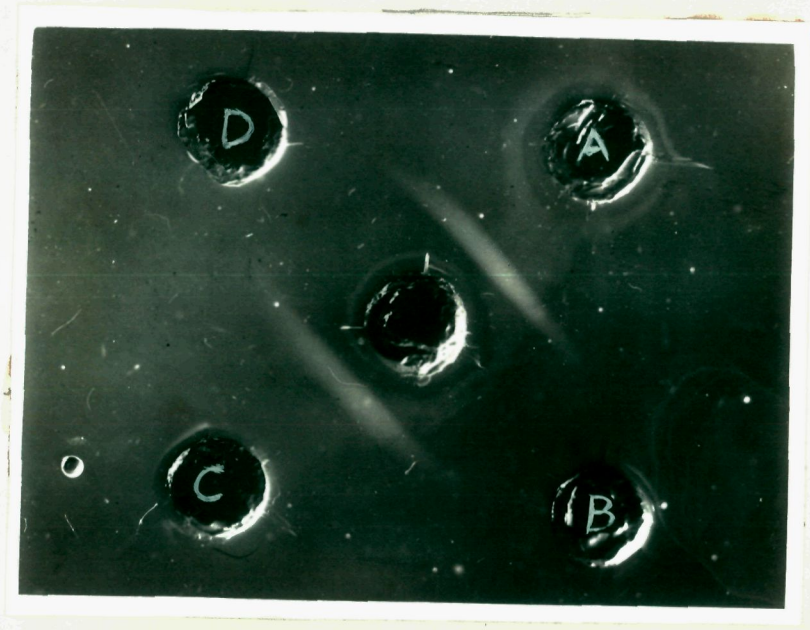


Figure 32. Rate of tryptic digestion of native and irradiated tyrosinase in presence and absence of psoralen. The concentrations of tyrosinase and psoralen were 750 µM and 12.5 µg/ml, respectively. The curves are (—○—) non-irradiated tyrosinase; (—●—) irradiated tyrosinase; (—△—) non-irradiated tyrosinase in presence of psoralen; (—▲—) tyrosinase irradiated in presence of psoralen and (—⬡—) trypsin control. Irradiation dose was 7.5×10^{19} Photons/cm^2.

Figure 33. Immunodiffusion pattern of native and irradiated tyrosinase in presence and absence of psoralen. Antigen solutions were kept in peripheral wells and the antiserum in the central well. The precipitin lines were allowed to develope for about 24 hours. (A) native tyrosinase; (B) irradiated tyrosinase; (C) tyrosinase in presence of psoralen and (D) tyrosinase irradiated in presence of psoralen. Irradiation dose was 7.5×10^{15} Photons/cm^2.


D
A
C
B

IV. DISCUSSION

1. Interaction between Psoralen and Tyrosinase in Absence of UV Light:

It is evident from the results that psoralen interacts with the enzyme. Exposure of chromophoric residues in lower dielectric solvents results in a decreased (quenching) fluorescence of the chromophore; since psoralen is hydrophobic in nature the decrease in fluorescence of enzyme indicates the possible involvement of aromatic amino acid residues in the interaction of tyrosinase with psoralen. Our preliminary studies on interaction of model compound (tryptophan) with psoralen indicated a significant quenching in the fluorescence of chromophore (142). The association constant was determined to be 8.4×10^6 M^{-1} which convincingly indicates that the complex of psoralen and tyrosinase is relatively quite stable. The value of free energy, i.e., the available energy required for the formation of psoralen - tyrosinase complex, was calculated to be -9 Kcal/mole which also supports the above contention. The effect of psoralen concentration on the enzyme molecule with respect to its fluorescence characteristic was also studied. Maximum quenching in fluorescence of enzyme was at a concentration of 24×10^{-5} M. The molar ratio between psoralen and tyrosinase was determined to be 77 at pH 6.5, indicating thereby that 77 moles of psoralen interact with one mole of tyrosinase.

The effect of pH on the complex formation between psoralen and the enzyme indicates an increase in molar ratio with increase in pH. Since ionization constant of imidazole group of protein molecule is near 6, the maximum binding of psoralen at pH 6.5 can be ascribed to possible involvement of histidine residues in the binding process.

It is of interest to see the effect of psoralen concentration on both the cresolase and catecholase activities. At pH 6.5 (the optimum pH for its biological activity) the increase in both the activities with increase in psoralen concentration suggests that the binding of psoralen to the enzyme decreases the activation energy of the reaction. It may be postulated that psoralen may act as a stimulator for the catecholase and cresolase activities of tyrosinase. Such stimulator or activator can be suspected in proteins having subunit structure (58,82,140). It seems, therefore, that the activation of tyrosinase activities by psoralen is one of its important physiological effect at the molecular level.

In view of the observation that psoralen interacts with the tyrosinase, we also tried to show the nature of interaction of psoralen with the enzyme molecule. The salt concentration from 0.01 - 0.1 M does not influence the fluorescence of psoralen - enzyme complex. The results suggest the involvement of hydrophobic and short range electrostatic interactions; because this much of salt concentration is not expected to effect the latter interaction. The hydrophobic interactions on the other hand are known to be stabilized in a polar solvent; the stability being directly related to the polarity of the solvent. Under the present experimental conditions (a buffer containing 0.1 M salt), it is obvious to conclude that hydrophobic interactions may play a major role

in stabilising the psoralen - enzyme complex due to the presence of strongly hydrophobic furan and coumarin ring in the psoralen. In addition to molecular characteristic of psoralen, the possible involvement of aromatic amino acid residues of tyrosinase as aforementioned has also been shown. It is of interest to note that the biological activity of tyrosinase is not affected by ionic strength (0.01 - 0.1) in presence and absence of psoralen. It is obvious that the ionic strength employed was not sufficient to alter the integrity of the biologically active enzyme molecule.

Effect of urea on the conformation and biological activity of tyrosinase in presence and absence of psoralen was also studied. The biological activity of tyrosinase is completely lost in 8 M urea, which is in agreement with the result of Kertesz et al. (56). Increase in fluorescence intensity was taken as denaturant parameter. The loss in the biological activity of an enzyme molecule as a result of chemical denaturant normally results in the alteration in the unique biologically active conformation (7). In presence of psoralen, the effect of urea on molecular conformation and biological activity was significantly decreased which strongly suggests that the drug acts as a protector of the enzyme molecule under these conditions.

2. Interaction Between Psoralen and Tyrosinase in Presence of UV Light:

Since interaction of psoralen with tyrosinase induced by UV light was the object of the study, the fluorescence spectra of tyrosinase as a function of irradiation dose was measured. The relative fluorescence is increased with increase in irradiation dose without any characteristic shift in fluorescence maxima. A maximum increase of 34 per cent was

detected at the highest irradiation dose. This increase in fluorescence is attributed to unfolding of the molecule which results in the exposure of chromophoric residues to the high dielectric medium (water). It is of interest to report that psoralen does not act as a photo-sensitiser, because tyrosinase irradiated in presence of psoralen does not show any increase in fluorescence intensity.

The fluorescence spectra of psoralen exposed to different doses of irradiation show a significant shift in the characteristic maximum (450 nm) to 440 nm with an additional hump at 400 nm. The latter developed into a well defined peak, the fluorescence intensity of which increased with increasing irradiation dose. The photochemical transformation of psoralen into new compound showing fluorescence at 400 nm might be suggested. Ultraviolet induced splitting of psoralen into furoumaric acid and psoralen dimer has already been reported (47, 141).

In view of the observation that the interaction of psoralen with the enzyme without irradiation increases the biological activity, the effect of 365 nm light on biological activity of the enzyme in presence of psoralen was also investigated. Irradiation in presence of psoralen lowers the activity at a comparatively faster rate and it follows first order kinetics. However, the rate of loss in activity is increased which might be the result of increased binding of psoralen with the enzyme. Increase in binding of psoralen with tyrosinase as a result of irradiation has been clearly shown and will be discussed later. The results suggest that psoralen has no photooxidative effect on tyrosinase in presence of ultraviolet light. Several contradictory reports have appeared regarding the photosensitising behaviour of psoralen with biopolymers. However, Rodighiero and Santamaria (85) showed convincingly

that furocoumarins are completely lacking in photooxidative property when compared with other photodynamic substances like methylene blue, chlorophyll, bengal rose, erythrosin etc. The observations of these workers were further confirmed by Oginsky et al. (46,100) who showed killing effect of xanthotoxin on bacteria at 365 nm was independent of the presence of oxygen.

The loss in biological activity in presence of psoralen and subsequent irradiation, therefore, may be due to direct interaction between the two reactants. It has already been shown earlier that the binding of psoralen in presence of UV light increases the activity of enzyme, however, further increase in binding of psoralen as evident from the number of molecules of psoralen which binds with enzyme (an increase from 72 to 92) induced by irradiation bring some local conformational change very near to the active site of tyrosinase which finally results in the decrease in activity.

Kinetic Parameters in Presence and Absence of UV Light:

The ability of tyrosinase to catalyze two reactions (cresolase and catecholase) is an interesting property and has stimulated much research. Interest of several workers has centered to characterize active sites for the two substrates (Tyrosine and Dopa). In the present studies determination of Km values for tyrosine and dopa in presence and absence of UV light favours two independent active sites on the enzyme molecule. Similar conclusion have also been reported by Dressler and Dawson (35) and later by Shimao et al. (137). The Km value for Dopa was uneffected when tyrosinase was irradiated in presence of psoralen. However, for tyrosine the Km was decreased from 2.2×10^{-4} M

to 0.5×10^{-4} M. This observation unequivocally suggests two different active centres in the enzyme molecule. The radiation induced decrease of Km observed in the present studies implies that the irradiated enzyme binds the substrate more strongly than does the non-irradiated tyrosinase. Similar observations have been reported recently by Saito (132) on irradiated Lactic Dehydrogenase.

Since the role of thiol compounds on melanin formation has been repeatedly mentioned by several investigators (76,66,134,131), it was thought of interest to study the inhibitory effect of sulfhydryl compounds on tyrosinase activity in presence and absence of psoralen, with and without UV irradiation. Generally a decrease in extent of inhibition by thiol compounds was observed on irradiated tyrosinase, which might be speculated due to certain modification in the conformation of enzyme as has been revealed for several other proteins and enzymes (9). The extent of inhibition after irradiation of enzyme in presence of psoralen is not effected to an appreciable extent, small changes, if any can be ascribed within emperimental error.

In the present studies the Ki value has been increased after irradiation of the enzyme. The change in Ki value may be due to alteration near the site of inhibitor binding, consequently, leading to change in conformation of enzyme molecule. The determination of Ki in presence and absence of UV irradiation, with and without psoralen, for two potent inhibitors of tyrosinase (thiouracil and glutathione) indicated certain modification in enzyme molecule, thereby increasing the association constant of enzyme and the inhibitor. The Ki value for thiouracil in presence of psoralen after irradiation is not increased. The results favour the postulate that psoralen protects the enzyme

molecule from the damaging effects of radiation in such a way that the association constant for enzyme and inhibitor is not effected.

It has been suggested by number of investigators that the increased melanin pigmentation after psoralen administration followed by UV irradiation photooxidizes the sulfhydryl compounds leading to increased tyrosinase action and better pigmentation (53). It is interesting to report that after irradiation of thiouracil and diethyldithiocarbamate, the inhibiting capacity is not effected inspite of marked changes in their UV absorption spectra.

Conformation of Enzyme:

The polyacrylamide gel electrophoresis of tyrosinase samples treated under several experimental conditions show single and sharp band, which suggests that irradiation in presence and absence is a homogeneous event. It is interesting to note that relative mobility of the enzyme is increased after irradiation in presence and absence of psoralen; indicative of a increase in its net negative charge. The breakage of certain disulfide bridges most expected after irradiation (9) may contribute to additional negative charge on the protein molecule. It has also been shown in our laboratory that the sulfhydryl contents of tyrosinase are increased after exposure to UV light (136).

The possible conformational change in tyrosinase molecule induced by UV irradiation in presence and absence of psoralen, was studied by tryptic susceptibility of the enzyme. Increase in proteolysis has been used in recording conformational changes in proteins (59). The increase in proteolysis due to secile peptide bonds exposure, can be used as a

criteria of unfolding of molecule. In our studies a 12 per cent increase in proteolysis after irradiation of tyrosinase may be ascribed to conformational change in the enzyme. Interestingly, irradiation in presence of psoralen does not bring more proteolysis which further strengthen the protective effect on tyrosinase by psoralen from irradiation. The enhanced binding between psoralen and tyrosinase after irradiation (92 psoralen molecule/mole of enzyme) masks the sevile peptide bonds which inturn become inaccessible to trypsin attack.

Specific antibody and antigen interactions have been considerably used to differentiate the two protein molecules with respect to the antigenic determinants which are supposed to be involved in the binding of antigen to antibody. In case of enzymes it has been also tried whether or not the active site and antigenic site are similar. It should be mentioned that if both the sites are similar any change in local conformation will diminish the biological as well as antigenic activities of the protein molecule. Surprisingly, our results on immunological characterisation of unirradiated tyrosinase and irradiated in presence and absence of psoralen show a significant destruction in antigenic determinants which results in the loss of immunological activity. However, about 30 per cent biological activity remained after irradiation under similar conditions indicating that active site is different from the antigenic site in enzyme molecule.

From these studies it could be concluded that psoralen in presence and absence of UV light interacts with tyrosinase, however in presence of UV light the extent of binding of psoralen is increased. Tyrosinase - psoralen complex is stabilised by hydrophobic and electrostatic interactions; aromatic amino acid residues and imidazole group of

tyrosinase are probably involved in the binding.

Psoralen seems to induce the biological activity of enzyme in the native state of the protein molecule; irradiation of tyrosinase in presence of psoralen shows a considerable decrease in biological activity, however, it preserves the native conformation of enzyme from UV light. Preservation effect of psoralen is also supported from the present investigation where potent denaturant, urea, distrupts the conformation of tyrosinase to a lesser extent in the presence of psoralen.

Kinetic studies are strongly in favour of the fact that dual activity of tyrosinase resides at two different sites in the protein molecule. It may also be speculated that the sites are not very far from each other.

Results of inhibition of tyrosinase by photooxidised inhibitors as well as native inhibitors indicated clearly that UV irradiation does not prevents the inhibition. Some of the basic studies on the effect of UV irradiation on the conformation and integrity of the enzyme molecule suggest that UV irradiation damage is a homogeneous process, in both presence and absence of psoralen. Further, the conformational parameters measured in terms of the suceptibility of tyrosinase towards trypsin strongly suggest that psoralen certainly has a photoprotective effect.

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